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Isolation studies of Leptospira from rats in Andhra Pradesh

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Abstract
Isolation studies were carried out from reservoir hosts rats, and the rats were trapped from different geo-graphical areas of Andhra Pradesh. The rats were ethunized and kidneys (cortical part) were processed for isolation. A total of 299 kidney processed samples were inoculated into a selective EMJH liquid medium containing 5-flurouracil (100µg/ml). Out of 299 samples processed for isolation studies, only 5 isolates (1.67%) were recovered. Further, the recovered isolates were purified and maintained in EMJH liquid medium. Physio-chemical caracterization of isolates revealed the absence of growth at 13^0C, absence of the growth in the presence of 8-azaguanine and lipase activity on egg yolk agar medium indicated the pathogenecity of the isolates. On dark field microscopy, three of the isolates shown characteristic flexion, extension and rotational movements suggestive of leptospira where as the other two isolates shown sluggish motility. On Molecular characterization two of the isolates sent for sequencing were identified as *Leptospira nagouchi* & *L. illini* on 16SrRNA PCR. This is the first report of isolation studies in rats from Andhra Pradesh and isolation studies from rats help in finding epidemiological link between animals, humans and rats (reservoir hosts).

Keywords: Rats, Isolation, EMJH liquid medium, 16SrRNA PCR

Introduction
Rodents are carrier of Leptospires throughout the world and important reservoirs of infection for man and domestic animals [1, 2]. The prevalence of Leptospirosis in tropical and subtropical countries is common because the countries like India have an ideal niche for the transmission of Leptopsires. The agriculture field, especially paddy and grain cultivating fields, are the most favorable places for their survival and procreation. The rodent population is high in agriculture fields because they can get their food adequately [3]. The rat is the primary carrier for the spreading leptospira through their excrement especially in the rice fields. Leptospires are spread by the contaminated urine of the infected rodents to the cattle through their grazing fields. This greatly increases the possibilities of spreading leptospires to the rice field workers [4, 3].
In rodents, especially the common rat (*Rattus norvegius*) and other reservoir species, persist indefinitely in the convoluted tubules of the kidney without causing apparent disease, and are shed into the urine in massive numbers [5]. *Rattus rattus* wroughtonihinton, *Rattus rattus* rufesens, *Bandicola indica* and *Bandicola bengalenses* are the rodent species involved in spreading Leptospirosis to humans and livestock in India [6].
Diagnosis can be made by serological identification using the microscopic agglutination test (MAT) and serovar level identification using specific monoclonal antibodies by further improved technique. Nowadays, the molecular diagnosis will be available and it is a convenient and simple method to perform compared to the earlier techniques. However, isolation of the organism is the most appropriate diagnosis compared to all other techniques though it is laborious and time taking. In Andhra Pradesh, so far no data related to isolation and identification of leptospires from reservoir hosts, rodents are available. Hence, in the present study, an attempt was made for isolation and identification of leptospires from rats to find out the epidemiological link between animals, humans and rats.

Materials and Methods
Collection of samples (Rats)
A total of 299 during the period from February, 2007 to December, 2009 Two hundred and ninety nine) rats were trapped in different areas in and around Tirupati (Table 1).

(Out of 299 samples, 20 rat samples from R.S. Junction, 22 samples of rats near Govindarajaswamy temple, 38 rat samples from Railway station and 19 samples of rats from Bus station, 15 samples of rats from Bhavani Nagar, 185 rats around Medical College and brought to the laboratory for leptospira isolation.

Isolation of leptospira

Processing of samples i*n rats*

A total of 299 rats collected from different areas of Tirupati were euthanized using chloroform and sacrificed. kidneys (Cortical part) were collected aseptically and made into 10 percent homogenous suspension with sterile PBS. 0.5ml of the suspension was inoculated into 5ml of EMJH liquid medium. The inoculated medium was incubated at 29 ^{O}C ± 1^{O}C in a BOD incubator and screened for the presence of leptospires at weekly intervals for a maximum of 6 weeks.

Purification of isolates

Filtration

Leptospira isolates thus obtained were purified by filtration through cellulose membrane filters with a pore diameter of 0.22µm (M/s. Sortorious, India) and cultured freshly into the EMJH liquid medium from the filtrate.

Use of selective media

Leptospira isolates were sub cultured into a medium containing 5-flurouracil (100µg/ml) to inhibit the growth of others and selectively grow the leptospires.

Dark field Microscopy

100µl of 5-7 day old well grown leptospira culture was taken on a clean, grease free microscopic glass slide. Cultures were examined under 20X magnification of a dark field microscope to study the characteristic motility of the leptospires.

Staining of Leptospires:

Place a loop of cultural smear on the slide and air dried. Then the slides are shed with a leptospira PD Admas staining kit (As per the kit protocol). Finally air dried the smear and examined under high power (40x) and later under an oil immersion (100x) microscope. Leptospires appeared as brownish black structures against yellowish background. Care should be taken to differentiate between fibrous and debris materials.

Physico-chemical characterization

Based on morphological characteristics, it is difficult to differentiate the pathogenic and saprophytic organisms. The recovered leptospiral isolates were subjected to physico-chemical characterization to differentiate pathogenic from non pathogenic leptospires.

The growth at 13 ^{O}C

Inoculated 0.05µl of well grown leptospiral isolates into 5ml of EMJH medium in duplicates. Similarly 0.05µl of known pathogenic and saprophytic strains of leptospires into 5ml of EMJH medium as controls in duplicates. Incubated one set at 13 ^{0}C and the other set at 30 ^{0}C. The growth was checked twice a week by a dark field microscope for up to 21 days.

Growth in the presence of 8-Azaguanine

Prepared 8-Azaguanine by dissolving 225 µg /ml and sterilized by autoclaving. Added 0.5ml of sterile 8-azaguanine solution to 4.5ml of EMJH medium and mixed thoroughly. Then inoculated the tubes with 0.05µl well grown leptospiral isolates under test and the tubes without 8-Azaguanine serve as control. Incubated the tubes both test and controls at 30^{0}C. Growth was checked weekly twice under the Dark field microscope for up to 21 days.

Reaction on Egg-yolk agar

Five percent egg-yolk emulsion was added to sterile nutrient agar media having pH 7.2 to 7.4. Two ml of 6-8 day old leptospira cultures containing approximately 10^{6} leptospires per ml were spread on egg yolk agar plates and incubated at 30 ^{O}C to study the lipase activity of leptospira isolates.

Molecular Characterization

DNA Extraction from cultures

DNA was extracted according to the method of Boom *et. al.* 1990 [7]. 4 ml of well grown leptospiral cultures were centrifuged at 13,000rpm for 15min. The pellet was washed twice and re-suspend in 500 µl of solution I (Appendix I). Later 50 µl of lysozyme (5mg/ml dissolved in the solution I) was added and incubated at 37^{O}C for 15 min. Later added 50 µl of 10percentage SDS, 5 µl of proteinase K (10mg/ml) and incubated at 65^{O}C for 30 min. 40µl of 5M Nacl and 32 µl of CTAB Nacl were added and incubated at 65 ^{O}C for 30min. Later equal volumes of chloroform iso amyl alcohol (241) (approximately 677 µl) were added. Then the contents were vortexed and centrifuge at 13,000 rpm for 15min. The supernatant (300 µl approximately) was collected and 180µl of chilled ethanol was added. After gentle mixing the contents were kept at -70^{O}C for 3hrs. Finally centrifugation was done at 10,000 rpm for 30min to pellet down the DNA. Contents were decanted and the pellet was dried over night and reconstituted in 50µl of TE buffer and stored at -20^{O}C until used.

PCR to amplify 16SrRNA

16SrRNA amplification was carried out according to the method of Shukla *et al.* 2003 [8] with certain modifications.

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'

In brief PCR was carried out in a gradient thermal cycler (Eppendorf) for 30 cycles using a set of specific primers I and II using the following steps. Initial denaturation at 94 ^{O}C for 5 min, annealing at 63 ^{O}C for 45 sec, extension at 72 ^{O}C for 1min, followed by cyclic denaturation at 94 ^{O}C for 1min, final annealing at 63 ^{O}C for 15min and final extension for 15 min at 72^{O}C. Each 50 µl of reaction mixture consisted of 25mM $MgCl_2$, 200mM dNTPs, 10mM Tris Hcl, 50mM Kcl, 0.5U of Taq DNA polymerase, 20p moles each primer and 2 µl of template DNA .

Confirmation of PCR products

The PCR products were analyzed in 1% agarose gel in TAE buffer (1X) with molecular weight marker (1Kb DNA ladder, Genei). The amplified products were visualized under a U.V trans illuminator and documented using Gel Documentation system (Alpha linear). The size of expected amplified nucleic acid was 525bp.

DNA sequence analysis

Amplified PCR products (2 samples) of 16SrRNA were sent

to M/s. Bioserve Biotechnologies Pvt. Ltd, Hyderabad for sequencing. The results of the sequence were subjected to nucleotide sequence analysis and homology study using BLAST N Search (clustal W2).

Results

EMJH liquid media inoculated with samples collected from rats were screened for the presence of leptospires under a dark field microscope. Out of 299 samples subjected for isolation, only 5 samples were found to have the organisms suggestive of leptospira with 1.67% positivity. Then the isolates were subjected to membrane filtration using 0.22 micron membrane filters for purification. Later, sub culturing was done on EMJH medium regularly and finally maintained on EMJH semi-solid medium for further studies.

Identification of leptospira

The leptospiral isolates (5) obtained during the isolation were maintained in EMJH liquid medium as well as the semisolid medium. The isolates were identified initially by dark field microscopy and by staining methods.

Dark field microscopy

Out of 5 isolates, three of them were actively motile, with characteristic motility of flexion, extension and rotational movements and two of the isolates were shown sluggish motility.

Fontana's staining

All the isolates showed characteristic morphology of spiral shape with hooked ends and seen as brown black with yellow color back ground (Fig.1) on staining.

Characterization of leptospira isolates

The isolates were subjected to physico chemical and molecular characterization.

Physico-chemical characterization

All the five recovered isolates were failed to grow at 13^0c and in the presence of an 8-azaguanine test (Table.2) and showed lipase activity on egg yolk agar plates indicating pathogenicity (Fig.2).

Molecular characterization

After physico-chemical characterization all five isolates were subjected for molecular characterization using 16SrRNA-PCR (Table.3)

16SrRNA amplification

The DNA extracted from all the 5 lepospiral isolates recovered from rats were subjected to 16SrRNA amplification. The results are shown in the Table.3 & Fig.3. The primers used in the study amplified the expected gene and yielded 525bp product which is specific to leptospira from all the isolates.

DNA sequence analysis of selected amplicons of leptospira organisms recovered from Bioserve Pvt. Ltd, Hyderabad was subjected to BLAST analysis and the results of the study indicated that the isolate RR1 obtained from rat found to have 93 percent homology with *L. noguchi* serovar panama strain and RG1 obtained from kidney sample of rat found to have 98 percent homology with *Leptonema.illini*.

Discussion

Leptospirosis can be diagnosed by several laboratory methods, but the isolation of leptospira allows the definite diagnosis of individual infections. It helps to study the molecular epidemiology of the disease and to develop a suitable vaccines in control of the disease. In AP, no data related to the isolation of leptospires from reservoir hosts like rodents are available. Hence, an attempt was made for isolation of leptospires from rats to find out the epidemiological link between animals and rats.

EMJH liquid medium with Tween 80, antibiotics and 5-flurouracil was used as selective media for isolation and maintenance of leptospira. Similarly, EMJH liquid media with Tween-80, antibiotics were also used by the earlier workers [9-14] for isolation and maintenance of leptospires in their studies. Schonberg, 1981 (15) reported the harmful effect of antibiotics on leptospiral growth after 48hrs incubation. However, the antibiotic effect on the 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 and leptospira was isolated from five of the 299 samples cultured. During the study, leptospiral organisms were observed in EMJH medium between 12-14 weeks of inoculation. Vijayachari *et al*, 2007 (16) and Gibson *et al*, 2008 [17] also observed leptospira between 12 to 14 weeks. Keeping in view of the slow growth of leptospira, the WHO, 2003 [18] suggested an observation period of 4 to 6 months for isolation of leptospira. The isolates grown in the EMJH liquid media were initially tested under dark field microscopy and noticed under dark field microscopy. The organisms were actively motile. The characteristic motility of flexion, extension and rotational movements was observed under a dark field microscope suggestive of leptospires.

Gangadhar and Rajasekhar, 1998 [19] also used ADMAS staining kit and reported the presence of leptospira. Characterization of leptospiral isolates is an important tool for the identification of leptospiral strains. Leptospiral isolates that were confirmed conventionally were subjected to physico-chemical characterization and were assessed by studying the leptospiral growth at 13 ^{0}C, in the presence of 8-Azaguanine and reaction on egg-yolk agar to differentiate pathogenic from non-pathogenic leptospires. During the study all the five leptospiral isolates fail to grow at 13^0C and in the presence of 8-Azaguanine. All the isolates showed lipase activity on egg-yolk agar indicating the pathogenic nature of leptospira (Jhonson and Roagers, 1964 [20]; Johnson and Haris, 1967 [21]; Noubade *et al*, 2002 [22]; Vijayachari *et al*, 2003 [14] and Shukla *et al*, 2003 [8] also differentiated pathogenic leptospires from non-pathogenic by growing at 13 ^{0}C, studying the effect of 8-azaguaine and lipase activity.

Leptospiral isolates were subjected to 16SrRNA PCR amplification for specific identification of leptospires by amplification of the conserved gene of 16SrRNA using specific primers (primer-I & primer-II) for identification of pathogenic leptospires. The selected amplified PCR products of the leptospiral isolates were sent for sequencing analysis (Bio-serve, Hyderabad). RR1 isolate had 93.00 percent homology with *L.noguchii* stain panama. RG1 isolate showed 98.00 percent homology with *Leptonema illini*. Several workers used 16SrRNA for the identification of pathogenic leptospires. (Merien *et al*, 1992 [23]; Shukla *et al*, 2003 [8];

Turk *et al*, 2003 [30]; Wangroongsarb, 2005 [25] and Djadid *et al*, 2009 [26]. *Leptonema illini* was also reported from rats by Gangadhar *et al*, 2000 [13]. *Leptonema illini* was condidered as non –pathogenic strain [27]. However, Hlavaz and Bazovska, 1974 [28] and Gangadhar *et al*, 2005 [29] reported the role of leptonema in inducing the pathogenecity.

In conclusion, this is the first report of isolation studies of leptospira from AP. However, a further detailed study is required in molecular epidemiology to known the prevailing serovars in rats, the source for transmission to animals and humans.

Table 1: 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
		299	**299**	

Table 2: Growth (Physico-chemical characterization) of leptospiral isolates

S. No	No. of samples tested	No. of samples recovered	Growth at		Growth with 8-Azaguanine	Growth without 8-Azaguanine	Controls			
			13 ^{0}C	30 ^{0}C			Reference pathogenic	Reference non-pathogenic		
							13 ^{0}C	30 ^{0}C	13 ^{0}C	30 ^{0}C
1.	299	5	-	+	-	+	-	+	+	-

Table 3: Results of 16SrRNA PCR of Leptospiral isolates from rats

Leptospiral isolates	Samples		Molecular diagnostic test
	Source	Type of material	16SrRNA
RR1	Rat	Kidney	+
RG1	Rat	Kidney	+
RR2	Rat	Kidney	+
RG2	Rat	Kidney	+
RG3	Rat	Kidney	+

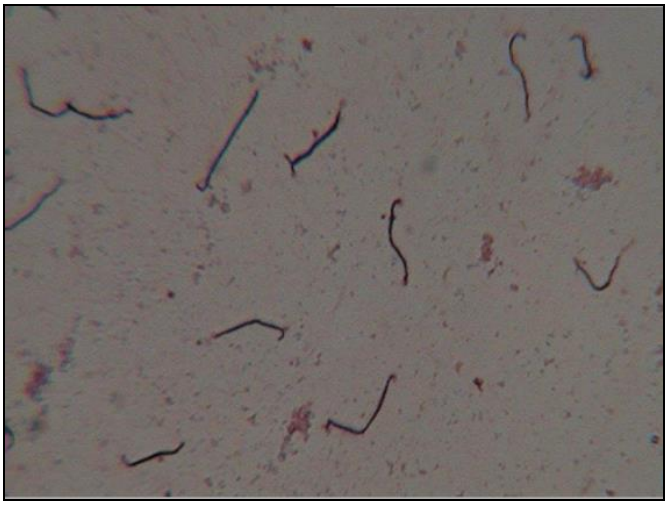
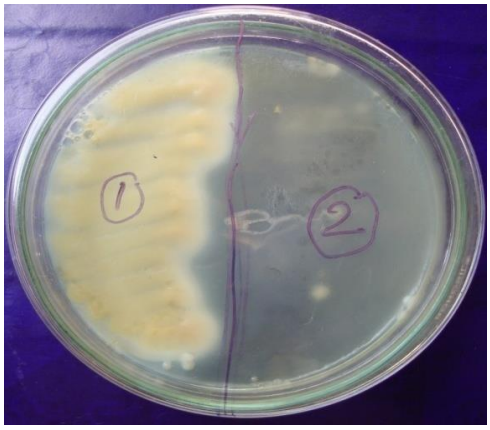


Fig 1: Fontana's staining of leptospira isolate (RR1)



1. Test: Leptospira isolate (RR1)
2. Negative control: Negative control (Non-pathogenic patoc-I)

Fig 2: Lipase activity of leptospira isolate (RR1) on egg yolk agar

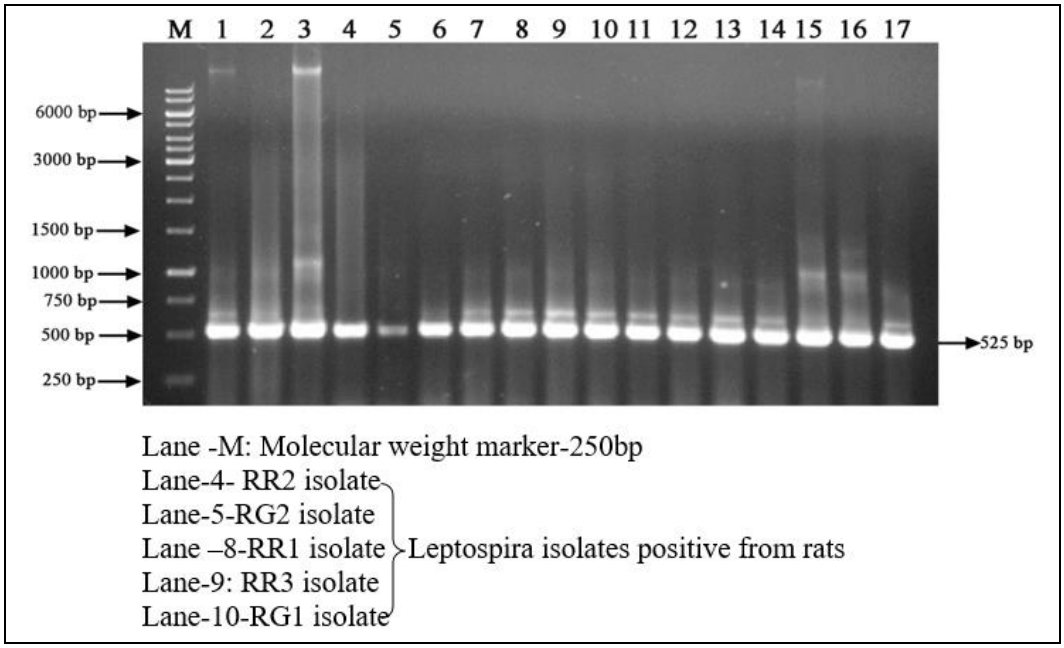


Fig 3: 16SrRNA PCR for leptospiral isolates from rats

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