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Laboratory diagnosis of leptospirosis in rodents

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Abstract
The present study was conducted to detect the carrier status in rodents by conventional and molecular methods in addition to the seroprevalence in rodents was identified by microscopic agglutination test. Rodents were trapped from Madras Veterinary College vicinity and human dwellings of Kattupakkam. Samples were collected under aseptic condition from rodents which consists of serum (30), urine (10) and kidney tissues (30).Urine and serum samples were screened by dark-field microscopy (DFM), out of 10 urine samples 4 (40%) and out of 30 serum samples 10 (33.3%) were found positive. Out of 30 kidney tissue samples 16 (53.3 %) were found positive by Polymerase Chain Reaction (PCR). The PCR amplicon size was 243 bp and 462 bp was seen on agarose gel electrophoresis with *lep* gene and *lipl32* gene respectively. The overall seroprevalence of leptospirosis in rodents detected by the presence of Leptospira antibodies in sera by Microscopic Agglutination Test (MAT) was 56.6 per cent. The major serogroup found was *autumnalis* followed by *grippotyphosa*, *icterohaemorrhagiae* and *pomona*. Sequencing was carried out for leptospire isolate from rodent kidney sample and found similarity in the sequenced product with other pathogenic serovars. The study concluded that rodents play an important role in transmission of the disease because they are caught from human vicinity so necessary precautions should be taken to avoid major public health risks.

Keywords: Leptospirosis, rodents, PCR, MAT, seroprevalence

1. Introduction
Rodents are one of the most common carriers for spreading of *Leptospira* among all mammals and were first confirmed by isolation in Indian rats (Knowles *et al.,* 1932) [2]. Prevalence of leptospirosis in tropical and subtropical countries is common because the countries like India have an ideal niche for the transmission of leptospires. There are three species of rodents that are distributed worldwide and are commonly associated with leptospiral infection: *Mus musculus* (house mouse), *Rattus norvegicus* (brown rat) and *Rattus rattus* (black rat). All three species belong to the order Rodentia, family Muridae, and subfamily Murinae. In rodents, especially the common rat (*Rattus norvegicus*) and other reservoir species, the organisms persist indefinitely in the convoluted tubules of the kidney without causing apparent disease, and are shed into the urine in massive numbers (Dutta and Christopher *et al.,* 2005) [1]. In rodents these bacteria multiply in their kidneys and are excreted via urine in the environment (Songer, 1983) [7]. The bacteria can survive for a long time in fresh water, damp soil, marshy fields and mud (Radostistis *et al.,* 2000) [3].The purpose of this survey was to determine the prevalence of *leptospira* infection in the rodents

2. Materials & Methods
Present study was undertaken at Madras Veterinary College. Thirty rodents were trapped from college premises and 10 urine, 30 serum and 30 kidney samples were collected. Rodents were anesthetized by means of carbon-dioxide inhalation. Blood samples for serology were collected from inner canthus of eye. The abdomen was then opened and kidney samples were collected. Urine sample from bladder under sterile conditions was collected using 2 ml syringe. Serum was separated from the clotted blood by centrifuging at 2000 rpm for 2 min and transferred to 2 ml screw- capped plastic vials and examined immediately for dark field microscopy and further stored at – 20 ºC.

2.1 Dark field microscopy: Urine and serum samples collected were centrifuged at 12000 rpm and the sediment was used to form wet mount preparation and was examined under dark field microscope.

2.2 Microscopic agglutination test: A panel of six serovars was used for the MAT (*autumnalis*, *canicola*, *grippotyphossa*, *icterohaemorrhagiae*, *pomona* and *pyrogens*). MAT was conducted as per the OIE (2004) in 96 well U bottom titration plates with dilution of 1:100. The highest dilution of serum showing 50 per cent reduction in number of free leptospires was considered as the end-titer.

2.3 Polymerase chain reaction: DNA isolation from kidney samples were done by using M/s Bio-Basic DNA extraction Kit The PCR reaction was carried out as per the method described by Suk *et al*., (2006).

Amplification of DNA was performed in a total volume of 25µl. Two different reaction mixtures were prepared with two different primers lep primer
(lep 1 TGGAGGAACACCAGTGGCGAAGGC and
lep 2 ACATGCTCCACCGCTTGTGCGGA) and
Lipl32 primer (Forward: TCCTGCCGTAATCGCTGAAATG and Reverse: CCTGGAATACCTGGTGGGAAAAG)

The reaction mixture contained 12.5 µl of master-mix,1 µl of each primer forward and reverse, 3 µl of DNA template and 7.5 µl nuclease free water. The samples were placed in automatic PCR Thermal Cycler (Eppendorf, Germany).

The cycling condition for PCR with *lep* primer is as follows: 95 ºC for 5 minute for melting, 40 cycles at 95 ºC for 1minute for denaturing, 52 ºC for 1minute for annealing, 72 ºC for 1 minute for extension, and 72 ºC for 5 minutes for the final extension sequence.

The cycling condition for PCR with *Lipl32* primer is as follows: 94ºC for 5 minute for melting, 30 cycles at 94 ºC for 1minute for denaturing, 54 ºC for 1minute for annealing, 72 ºC for 1 minute for extension, and 72 ºC for 7 minutes for the final extension sequence.

After amplification a 10 µl portion of each sample was subjected to electrophoresis on a 1.3% agarose gel at 100 V for 20 to 30 minutes until the tracking dye migrated more than two third of the length of the gel tray in the buffer and the results were documented in a gel documentation system (Gel Doc Mega). The PCR amplicon size was 243 bp and 462 bp was seen on agarose gel electrophoresis with *lep* gene and *lipl32* gene respectively. The positive PCR product was sequenced by using Lipl32 primer.

3. Results

Dark field microscopy (DFM) detected leptospires in 4 (40 per cent) of rodents urine samples and 10(33.3 per cent) in serum samples. Whereas PCR detected leptospiral DNA in 16 (53.3 per cent) rodent kidney samples of the total of 30 samples.

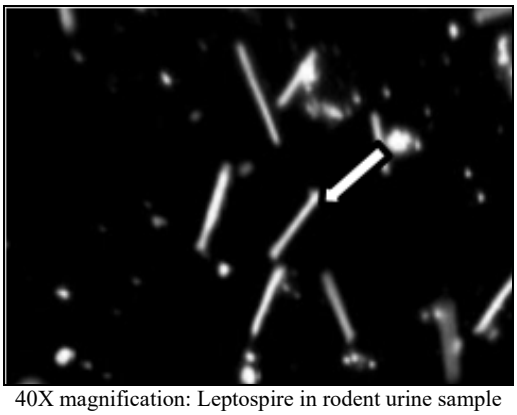
Seroprevalence of leptospiral antibodies by MAT among rodents trapped from the premises of college revealed 17 (56.6 per cent) samples were positive by agglutinating with one or more of the four leptospira antigens tested, out of 30 sera tested. The major serogroup found in rodents was *autumnalis* (47.05%) followed by *grippotyphosa* (23.5%), *icterohaemorrhagiae* (23.5%) and *pomona* (5.88%) at 1:100 titre.

3.1 Sequencing of PCR product

3.1.1 PCR with 462 bp LipL32 primer

The tissue sample (rat kidney) for pathogenic leptospira was subjected to PCR with 462 bp LipL32 primers which was positive for Leptospira. An amplicon of 462 bp was obtained.

The amplicon was sequenced and the sequence obtained was analyzed using BLAST to check for the homology (www.ncbi.nlm.gov/blastn). The sequence was found to have 100 per cent homology with accession numbers (KC800993.1), (KC800990.1), (KC800989.1), (JQ013520.1), (JQ013518.1), (JN886739.1), (CP001221.1), (GU592524.1).



40X magnification: Leptospire in rodent urine sample

Fig a: Dark field Microscopy

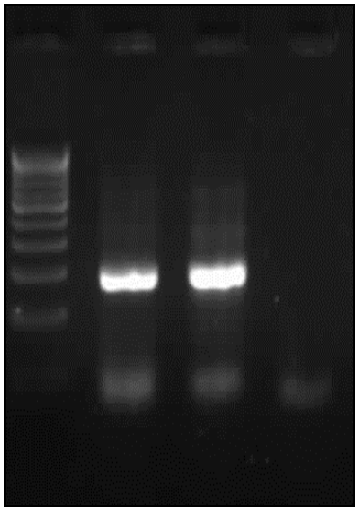


Fig b: Gel Electrophoresis of PCR Products with *Lep gene*, Lane L: Marker 100bp, Lane 1: Rat kidney sample, Lane 2: Positive control, Lane 3: Negative control

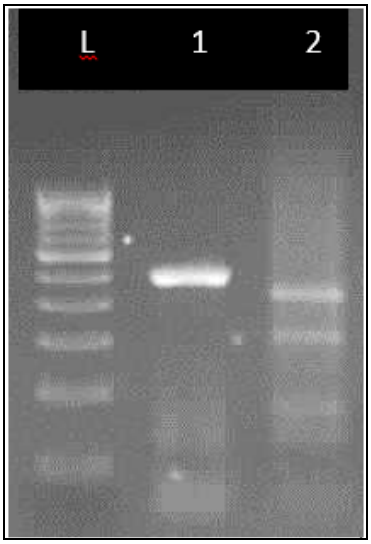


Fig c: Gel Electrophoresis of PCR Products with *Lipl 32 gene*, Lane L: Marker 100bp, Lane 1: Rat kidney sample, Lane 2: Positive control

4. Discussion

The DFM detected 40 per cent (4 out of 10) positives in urine samples and 33.3 per cent (10 out of 30) positives in sera samples. Vinodkumar *et al*. (2011) [9] revealed the presence of leptospiral antigens in 17.65 per cent in urine samples of rats by DFM. However, the sensitivity of this method is low, approximately 1×10^4 leptospires/ ml are necessary to observe one cell per field.

In order to detect carrier status in rodents, kidney tissues was selected as the sample of choice. In the present study 53.3per cent positivity (16 out of 30) was detected by PCR. Rahelinirina *et al*. (2010) [6] reported 50 per cent (25 out of 50) positivity in the kidney tissues of rodents by PCR. Priya *et al*. (2007) [4] observed a positivity of 46.15 per cent (6 out of 13) in the kidney tissue of rodent. In the present study, the high positivity may be because the cortical area was selected for extraction of DNA and this may be a suitable site for localization of leptospires.

The overall prevalence of leptospirosis in rodents detected by the presence of Leptospira antibodies in sera was 56.6 per cent. Priya *et al*. (2007) [4] revealed the presence 58 per cent anti-leptospiral antibodies in the sera of field rats whereas Vedhagiri and Natarajaseenivasan (2010) [8] found a seroprevalence of 51.4 per cent in rodents. A high seropositivity among rats showed that they are major contributory source for the spreading of leptospires.

In the present study the major serogroup found in rodents was *autumnalis* (47.05%) followed by *grippotyphosa* (23.5%), *icterohaemorrhagiae* (23.5%) and *pomona* (5.88%) at 1:100 titre. Similarly Pargoankar (1957) found that the seropositivity in Tamil Nadu due to serogroup *autumnalis* was 52 per cent and Vedhagiri and Natarajaseenivasan (2010) [8] observed that the predominant serogroup were *autumnalis* (25.7%), *javanica* (14.3 %), *icterohaemorrhagiae* and *pomona* (5.7%). *Autumnalis* from a high proportion of rats identifies these rodents as a major natural carrier and shedder of leptospires and hence as an important source of infection to humans and other animals.

5. References

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