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The journal is official publication of the Staff Society of Seth G. S. Medical College and K. E. M. Hospital, Mumbai, India and is managed, printed and distributed by Medknow Publications. Issues are published quarterly in the last week of January, April, July and October.

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The Journal is printed on acid free paper.

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Field rats form a major infection source of leptospirosis in and around Madurai, India

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ABSTRACT

Aims: To determine the seroprevalence of leptospires and to isolate *Leptospira* spp. from field rats and bandicoots in and around Madurai.

Materials and Methods: Thirteen rats and five bandicoots were trapped alive from fields in and around Madurai. Blood samples were tested for anti-leptospiral antibodies by microscopic agglutination test while the urine and kidney samples were used for isolation of leptospires. The isolated leptospires were tested for pathogenic status (13°C test and PCR) followed by serological and genetic characterization.

Results: Serology revealed the presence of anti-leptospiral antibodies in 58% (7/12) of field rats and leptospires were isolated from two urine and six kidney samples. The bandicoots were negative in both serology and culture. Analysis of the isolates from field rats revealed that all the isolates were pathogenic except for one, which was further confirmed by serological and genetic characterization. Six of the seven pathogenic isolates were identified as *L. interrogans* serogroup Autumnalis serovar Akiyami A and one as *L. borgpetersenii* serogroup Javanica serovar Veldrat Batavia 46.

Conclusions: Serology and isolation reveals that field rats are major natural carriers and shedders of leptospires in and around Madurai.

Received : 14-08-2007
Review completed : 19-10-2007
Accepted : 01-11-2007
PubMed ID : ????

J Postgrad Med 2007;53:236-40

KEY WORDS: DNA typing, isolation of leptospires, leptospiral serology, leptospirosis, rats, serotyping

Leptospirosis is a widespread zoonosis caused by pathogenic spirochaetes of the genus *Leptospira*. It assumes zoonotic importance since rodents and certain species of domestic animals such as dogs, pigs and cattle are known to be natural carriers of various serovars thus forming infection reservoirs in the transmission of infection to other animals and man, especially to professionally vulnerable groups such as sewage workers, farmers, butchers and veterinarians.^[1] In humans, the severity ranges from a mild flu-like illness to a severe infection with renal and hepatic failure, pulmonary disease, myocarditis, hemorrhages and death.^[2] Ocular manifestation (uveitis) of systemic leptospirosis occurs at a later stage and has been reported even after one year of systemic illness.^[3] This form of uveitis is referred to as leptospiral uveitis.^[4]

There are several reports indicating more than 30% seropositivity for leptospirosis in Southern India including Chennai and Madurai in Tamil Nadu.^[5-8] In 1993, after a heavy rainfall and unexpected flooding, many residents in and around Madurai developed an acute febrile illness. After the subsidence of fever, many of these patients developed uveitis. Following this, extensive studies on the seroprevalence of leptospirosis among humans were done at Aravind Eye Hospital, Madurai and Australis, Autumnalis, Icterohaemorrhagiae and Louisiana were identified as the most common serogroups in this region. Till date, around 200 new cases (10% of total uveitis cases) are reported every year at the Uvea clinic, Aravind Eye Hospital.^[9] Madurai, situated on the banks of river Vaigai, has many rice fields on its outskirts. Leptospiral uveitis has often been

encountered in connection with rice harvesting and a major proportion of the uveitis patients were farmers.^[10-13] Since the epidemiology of leptospirosis of any region is best reflected by the serovars carried by the rodents in that region, the present study was undertaken to determine the seroprevalence of leptospirosis and to isolate *Leptospira* spp. from rats and bandicoots in and around Madurai. Serotyping and DNA typing of the isolates were carried out to identify the infecting serovar and the results were compared with the most prevalent infecting serovar among leptospirosis patients in this region.

Materials and Methods

Samples: Thirteen rats (presumably *Rattus rattus*) and five bandicoots were trapped alive from fields in and around Madurai. All animals had a healthy appearance. The animals were anaesthetized by means of chloroform inhalation. Blood samples for serology were collected by cardiac puncture. The abdomen was then opened and urine was collected from the bladder under sterile condition using a 1 ml syringe and finally one kidney was removed. The urine and kidney were used for isolation of leptospires. The study was carried out with the permission from the Government Multispeciality Veterinary Hospital, Madurai.

Serum: Blood (5 ml) was collected in vacutainer tubes and allowed to clot at room temperature. After centrifuging for 20 min at 2000 rpm, serum was separated, aliquoted and stored at -20°C. It was not possible to collect serum from rat number 3.

Microscopic agglutination test (MAT): A panel of 18 serovars was used for MAT [Table 1]. MAT was performed as per the standard procedure starting with a serum dilution of 1:20 up to 1:20480.^[14] The highest dilution of serum showing 50% reduction in free-moving leptospires under dark field microscope was considered the end-titer.

Culture medium: For isolation, medium of Ellinghausen-McCullough as modified by Johnson and Harris (EMJH)

supplemented with 200 µg/ml 5-fluorouracil (EMJH-FU) was used.^[2,15,16]

BSA-Tween 40/80 medium containing 1% rabbit serum and 100 µg/ml 5-FU, 10 µg/ml rifampicin and 2 µg/ml amphotericin B was a kind gift from Dr. Brem, Germany and Dr. Ellis, United Kingdom.

Culture: The urine and macerated kidney samples were inoculated in EMJH-FU medium within two hours after removal. The pH of the samples was alkalized by adding PBS of pH 8.

Kidney: Kidneys were rinsed with sterile distilled water and put in a sterile Petri dish. In the laminar hood, a piece of 0.5 cm³, containing mostly the cortex was cut and macerated using mortar and pestle. It was then suspended in 0.5 ml EMJH medium and 0.5 ml PBS and sodium hydroxide (pH 8.0). The suspension was then centrifuged for 5 min at 800 rpm to remove the tissue material. 0.5 ml of the supernatant was inoculated in 5 ml EMJH-FU medium and further two 10-fold dilutions were made from the initial inoculum to dilute potential growth inhibitor.

Urine: Urine was centrifuged for 15 min at 4000 rpm. Strongly acid urines were made alkaline by adding PBS, pH 8.0. 0.5 ml of the sediment was inoculated in 5 ml EMJH-FU medium and two 10-fold serial dilutions from the initial inoculum were made in the same culture medium.

The samples were incubated at 28-30°C up to four months and checked regularly for the presence of Dinger's disc and for live leptospires under dark field microscope. The cultures were checked once a week in the first month, once in two weeks in the second and once in a month in the third and fourth months.

Contaminated positive cultures were subcultured in BSA-Tween 40/80 medium.

Microscopy and staining

The morphological characters of the isolates were studied using dark-field microscopy and modified silver staining technique.^[17]

13°C test: To determine the pathogenic status of *Leptospira* isolates, growth rates in EMJH medium at 13°C and 30°C were determined according to Johnson and Harris (1967).^[16]

Briefly, 0.1 ml cultures were inoculated into 10 ml EMJH medium in four tubes and duplicates incubated at 13°C and at 30°C to determine the growth rates at a low and high temperature respectively. *Leptospira* growth was determined by dark field microscopy up to 12 days. Pathogenic strain Wijnberg and saprophytic strain Patoc I were included in the test for comparison of growth responses.

Serological typing: MAT with group sera and monoclonal antibodies: To identify the isolate up to serogroup status, MAT was performed following standard procedure using a

Table 1: Panel of leptospiral reference strains used for microscopic agglutination test

| Serogroup | Serovar | Strain |
|---------------------|---------------------|--------------------|
| Australis | Australis | Ballico |
| Australis | Bratislava | Jez-Bratislava |
| Autumnalis | Autumnalis | Akiyami A |
| Ballum | Ballum | Mus 127 |
| Canicola | Canicola | Hond Utrecht IV |
| Grippotyphosa | Grippotyphosa | Moskva V |
| Hebdomadis | Hebdomadis | Hebdomadis |
| Icterohaemorrhagiae | Copenhageni | Wijnberg |
| Icterohaemorrhagiae | Icterohaemorrhagiae | RGA |
| Javanica | Javanica | Veldrat Batavia 46 |
| Louisiana | Lanka | Le 740 |
| Mini | Mini | Sari |
| Pomona | Pomona | Pomona |
| Pyrogenes | Alexi | HS 616 |
| Sejroe | Hardjo-bovis | Lely 607 |
| Sejroe | Hardjo-prajitno | Hardjoprajitno |
| Semarang | Patoc | Patoc I |
| Tarassovi | Tarassovi | Perepelitsin |

panel of 41 anti-*Leptospira* rabbit antibodies.^[14] Isolates were further typed at the serovar level by performing MAT with a panel of monoclonal antibodies (mAbs) that characteristically agglutinate serovars from the serogroups Autumnalis (F64C2, F64C6, F64C7, F64C8, F64C10, F65C3, F69C2, F69C8, F69C9, F69C10, F69C11, F69C12, F69C14, F69C15) and Javanica (F12C3, F20C3, F20C4, F70C20, F98C4, F98C5, F98C8, F98C12, F98C17, F98C19 and F98C20).^[18]

Genetic characterization: Strains and isolates were grown at 30°C in EMJH medium and harvested by centrifugation during the late logarithmic phase. The DNA was isolated as described by Boom *et al.*^[19] PCR was performed on the DNA extracts using the following sets of primers: (i) PCR with primer pairs Lepat1/2 and Saprol/2 according to the procedure described by Murgia *et al.*^[20] Lepat1/2 is specific for pathogenic leptospires while saprol/2 specifically amplifies DNA from saprophytic leptospires. And (ii) G1/G2 and B64-I/B64-II.^[21] Primer set G1/G2 amplifies DNA from all pathogenic species except *L. kirschneri* and primer set B64-I/B64-II specifically amplifies DNA from species *L. kirschneri*. The PCR conditions and controls were done as previously described.^[21,22] The PCR products were further analyzed by agarose gel electrophoresis on gels containing 1.5% agarose, stained with ethidium bromide using standard procedures and subsequently judged by eye under UV illumination.

Southern blot analysis of G1/G2-generated PCR products was performed with DIG-labelled probe G195-28 according to Bal *et al.*, with a modification that hybridization of the probe was done at 38°C.^[22]

For sequencing, DNA concentration of PCR products were adjusted in the range of 10-20 ng per reaction and applied to the sequence reaction using the BigDye® Terminator V1.1 Cycle Sequencing Kit (Applied Biosystems, United Kingdom) and subsequently analyzed on an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, United Kingdom). The DNA sequence clustal alignments were done using the LaserGene software package (DNASTAR). Species determination was done on basis of a higher sequence identity of PCR products from strains within a single species compared to sequence identities from strains belonging to different species.^[21,23]

Results

Serology

In the MAT, none of the bandicoots were positive for leptospirosis.

Seven out of 12 (58%) field rats were seropositive by MAT. According to highest titers, one serum sample had a titer of 1:20 against the Australis group, two sera had a titer of 1:40 against the Hebdomadis and Tarassovi groups and four sera had titers ranging from 1:80 to 1:1280 against serogroups Australis (1x), Autumnalis (2x) and Hebdomadis (1x). Cross-reacting serogroups included Javanica, Hebdomadis, Australis and Tarassovi. No reactions were detected against serogroups Canicola, Grippotyphosa, Icterohaemorrhagiae, Mini and Sejroe.

Isolation

Leptospira were isolated from two urine samples (rats numbered 11 and 13) and six kidney samples of rats (rats numbered 4, 5, 8, 10, 11 and 13). No isolates were obtained from urine and kidney samples of bandicoots.

Morphology and staining

All isolates showed typical morphology and characteristic motility of the genus *Leptospira* under dark-field microscopic examination. The cells were stained well by silver impregnation technique [Figure 1].

Pathogenic status

All isolates from the kidney or urine of rats numbered 4, 5, 8, 10, 11 and 13, together with the control strains Wijnberg (pathogenic) and Patoc I (saprophytic) did grow well at 30°C. However, at 13°C only Isolate 5 and Strain Patoc I did grow while growth of Isolates 4, 8, 10, 11 and 13 together with Wijnberg was severely hampered. This result is suggestive of a pathogenic status for Isolates 4, 8, 10, 11 and 13 and a saprophytic status for Isolate 5.

In the PCR and subsequent agarose gel electrophoresis analysis, the isolate from the kidney of rat number 5 (KR 5) gave a strong product with saprol/2 primer and only a very weak band with lepat1/2 primer, supporting its saprophytic status. All other isolates gave a strong signal with lepat1/2 primer and no signal with saprol/2 primer (results not shown). The DNA of all isolates amplified a 285 bp product in PCR using primer pairs G1/G2. The DNA from Isolate KR-5 gave a non-specific signal with B64-I/B64-II primer while all other isolates were negative. Thus none of the isolates belonged to *L. kirschneri*.

When applying Southern blotting on the G1/G2 generated products, all products except from KR-5 gave a positive signal with DIG-labeled probe G195-28, indicating that the G1/G2-generated amplicon from KR-5 represents an anomalous product. This finding confirms the results of the conventional 13°C test and pathogenic-specific PCR that all isolates except KR-5 are pathogenic leptospires.



Figure 1: *Leptospira*s stained by modified silver impregnation technique. Silver-stained isolate from a rat kidney showing leptospires as brown colored long curved organisms.

Serological characterization

MAT with 41 serogroup-specific sera followed by panels with mAbs characteristically agglutinating serovars of the serogroups Autumnalis and Javanica revealed that six isolates (isolates from urine of rats numbered 11 and 13 and from kidneys of rats numbered 4, 10, 11 and 13) belonged to serovar Autumnalis, strain Akiyami A and one isolate (Rat 8) belonged to Javanica, strain Veldrat Batavia 46 (results not shown). The isolate from Rat 5 was non-reactive with the polyclonal antibodies tested which further implicates that this is a saprophytic isolate.

Isolates and MAT

To determine homologous titers, rat sera were subjected to MAT using the isolates as antigen. Only the sera from Rats 10 and 11 produced titers. Agglutination titers against their own isolates did not differ significantly from those obtained with isolates from Rats 4, 8 and 13 [Table 2]. For Isolates 4 and 13 this was not unexpected as all four isolates, 4, 10, 11 and 13 were typed as the same serovar Autumnalis, strain Akiyami A. Isolate 8, typed as serovar Javanica, is not related to serovar Autumnalis. Therefore, the titers produced with Isolate 8 are rather due to exposure to serovar Javanica than to cross-agglutination of the sera. No agglutination titers were found with the sera from Rats 1, 2, 4 - 9, 12 and 13 using the isolates as antigen in the MAT. Moreover, field rats numbered 4, 8 and 13, did not show any serological proof of leptospirosis, including in the MAT with reference strains, though leptospire were isolated from their urinary tract or kidneys. None of the sera from the rats agglutinated with the *Leptospira* isolate from Rat 5, which is in accordance with the saprophytic status of this isolate.

Genetic characterization

Serogroup Autumnalis, serovar Autumnalis, strain Akiyami A belongs to species *Leptospira interrogans* sensu stricto while serogroup Javanica, serovar Javanica, strain Veldrat Batavia 46 belongs to *L. borgpetersenii*.

Sequencing of G1/G2-generated products and subsequent homology analysis were in concordance with this classification. The DNA sequences of the isolates from Rats 4, 10, 11 and 13 showed highest homology with sequences of reference strains

belonging to *L. interrogans* s.s. and the product of Rat 8 shared the highest sequence identity with sequences from reference strains of *L. borgpetersenii*.

Discussion

Rodents have been shown to be the main carriers of pathogenic leptospire and their local abundance can be an indicator of the potential transmission of *Leptospira* spp. to humans and livestock.^[24,25] In India, the natural leptospiral infection in rats was first documented in Kolkata, subsequently in Mumbai and in Kerala with the isolation of Autumnalis, Hebdomadis and Javanica in rats and bandicoots.^[26-28] Javanica was isolated in Karnataka and in Tamil Nadu, isolation of Autumnalis and Javanica was possible from rats, bandicoots and goats (unpublished observations).^[18,29] A 46% positivity of viable leptospire belonging to the serogroups Autumnalis and Javanica from the field rats in our study is consistent with these previous findings and indicates that these rats might represent the natural carriers of serovars from these serogroups.

Paragoankar (1957) demonstrated 11% seropositivity against *Leptospira* among rats in Andhra Pradesh but the seropositivity was 52% in Tamil Nadu mostly to serogroup Autumnalis.^[30,31] Earlier studies in Tamil Nadu have revealed high titers to Autumnalis followed by Icterohaemorrhagiae, Panama and Sejroe in patients with systemic leptospirosis.^[7,8] In patients with leptospiral uveitis, Australis, Autumnalis, Icterohaemorrhagiae and Louisiana have been identified as the most common serogroups.^[10-13] A 58% seropositivity with the highest titer to Autumnalis in rats in our study is in accordance with Autumnalis being a dominant infecting serogroup. The high seroprevalence and the presence of viable leptospire in field rats signify that these rats could be a major source of leptospiral infection to humans in Madurai and its surroundings.

The results of MAT and culturing revealed that Rats 4, 8, 10, 11 and 13 were asymptomatic carriers of pathogenic leptospire. Three of these five rats (60%) had a negative serology in the MAT. This phenomenon of isolation positive, serologically negative rodents has been observed repeatedly by different authors independently on the infecting serovars, indicating the limitations of serology to determine the carrier status of natural hosts.^[32]

Even though no isolation or seropositivity to leptospire was observed in bandicoots in this study, reports are available from India that they too are carriers of serogroups Autumnalis and Javanica.^[18,28] In a concomitant random serosurvey on cows and dogs in and around Madurai, we found 90% of the cows and 70% of the dogs to be seropositive. In cows there was a wide variety of reacting serovars. Notably serovars of the serogroups Mini, Sejroe, Hebdomadis, Grippotyphosa, Icterohaemorrhagiae and Pyrogenes agglutinated till high titers (reciprocal titers 1280 to 5120). In dogs, significant titers (≥ 160) were found against serogroups Autumnalis, Icterohaemorrhagiae, Sejroe and Canicola (unpublished observations). Agglutination and cross-agglutination with a variety of serovars was also observed in the sera of 12 rats. Apparently, there is a high *Leptospira*

Table 2: Reciprocal microscopic agglutination test titers on serum samples from 12 rats*

| Serum Rat number | Isolate from kidney | | | | | |
|---------------------|---------------------|---|-----|-----|-----|-----|
| | 4 | 5 | 8 | 10 | 11 | 13 |
| 1 | - | - | - | - | - | - |
| 2 | - | - | - | - | - | - |
| 4 | - | - | - | - | - | - |
| 5 | - | - | - | - | - | - |
| 6 | - | - | - | - | - | - |
| 7 | - | - | - | - | - | - |
| 8 | - | - | - | - | - | - |
| 9 | - | - | - | - | - | - |
| 10 | 640 | - | 320 | 640 | 320 | 640 |
| 11 | 320 | - | 80 | 160 | 80 | 160 |
| 12 | - | - | - | - | - | - |
| 13 | - | - | - | - | - | - |

*MAT was performed with a panel consisting of the rat isolates

infection rate between domestic and wild animals. We therefore hypothesize that humans in the region of Madurai are also at high risk for acquiring leptospirosis and subsequently leptospiral uveitis from various infection sources.

Isolation of leptospires belonging to serogroup 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. Future research will include isolation and typing of leptospires from human and animal patients to confirm Autumnalis as a common serogroup with rats a major source in the region.

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Source of Support: Aravind Medical Research Foundation, Madurai, India and KIT Biomedical Research, Amsterdam, The Netherlands.
Conflict of Interest: None declared.