Reservoir hosts of *Leptospira inadai* in India

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Summary
Isolation of *Leptospira* from the kidneys of *Rattus rattus wroughtoni hinton*, *Rattus rattus rufescens*, *Bandicota bengalensis* and *Bandicota indica* was attempted in Bangalore in southern India. In total, 296 spirochaetes were isolated from 1,348 kidney cultures (an isolation rate of 22%). A batch of fifty-six isolates from India was identified, based on serological and polymerase chain reaction analysis, of which twenty-three isolates were identified as *L inadai* by the World Health Organization/Food and Agriculture Organization Collaborating Centre for Reference and Research on Leptospirosis, in Brisbane. This is the first record of isolation of *L. inadai* from rodents. The preponderance of *L inadai* in four different species of rodents suggests that these animals could be the natural reservoir hosts of *L. inadai*, and raises a critical question as to the likely impact of this species of *Leptospira* on the renal carrier status of other *Leptospira* pathogenic to humans and animals in this part of India.

Virulence studies conducted at the University of Trieste in Italy, revealed that isolates of *L inadai* from India were moderately or totally serum resistant when subjected to a serum killing test. To establish the possible seroprevalence of this species in the population, the inclusion of *L inadai* in the battery of leptospiral antigens used for sero-epidemiological studies is recommended.

Keywords
Bandicota - Isolation - Leptonema - Leptospira inadai - Leptospirosis - Pathogenicity - Polymerase chain reaction - Rattus - Reservoir hosts - Serotyping.

Introduction
Leptospirosis is a world-wide zoonosis, with more than 250 serovars of *Leptospira* that are pathogenic to man and animals. Leptospires are generally carried in the kidney of natural reservoir hosts, such as rodents and domestic animals. Perpetual emergence of leptospirosis within a community is primarily influenced by the size and type of the rodent population, in addition to the occurrence of intermixing of different types of rodent, in an agrarian milieu where humans, rodents and other animals share common sources of food, water and shelter. In most parts of the world, the rodent reservoir hosts of various *Leptospira* species have been identified (10, 11, 13, 16, 18). However, only limited information is available in India (2, 5, 14, 17).

The present study reports field investigations which were undertaken to identify the rodent reservoir hosts of leptospires in and around Bangalore city in southern India.

Materials and methods

Source and identification of rodents
During a four-year study from 1994 to 1998, 1,348 rodents were trapped from 112 locations, including sixty-one human dwellings, forty-eight dairy farms, and one each of an animal feed plant, a laboratory animal house and a kennel. The representative rodent specimens were referred to the Zoological Survey of India, Calcutta, India, where 819 were identified as *Rattus rattus wroughtoni hinton* (R. hinton), 412 as...
and forty-two as *Bandicota bengalensis*. *Rattus* seventy-five as *Bandicota rattus rufescens*, *(R.* Ellinghausen McCullough Johnson Harris (EMJH) semi-solid maintenance of leptospires (3). The medium was stored at 4°C until required.

**Isolation of Leptospira**

The study included 1,348 rodents, 616 of which were trapped from dairy farms, 546 from human dwellings, eighty-six from the premises of a laboratory animal house, seventy-six from an animal feed mixing plant and twenty-four from a kennel. These rodent habitats were randomly selected for trapping based on the prevailing rodent infestation in these locations, so that rodents from diverse locations were available for study. The rodents were euthanised by placing the traps inside an airtight box containing a cotton wad soaked in chloroform (10 ml). After 15 min, the rodents were individually dipped in a jar containing 70% alcohol to decontaminate the external surface of the animals, and the necropsy was performed in a bio-safety laminar flow bench. The abdominal cavity was opened from the pelvic region through lateral incisions, cranially on both sides. The abdominal flap was then reflected backwards and the viscera were gently moved aside to expose the kidney. Holding the kidney with sterile flat tipped forceps, a sterile Fasteur pipette was used to horizontally pierce the cortex and the aspirated fragments were inoculated into EMJH semi-solid medium. Similarly, the cortical tissue from the other kidney was inoculated into the same culture tube. The inoculated culture tubes were routinely screened by dark field microscopy at weekly intervals, and when necessary, were stained by modified silver impregnation technique to demonstrate the leptospires, as an adjunct to the dark field microscopy. This staining technique, developed at the laboratory, all the isolates were initially screened against a reference panel of twenty-one serovars, using the microscopic agglutination test (MAT) to establish whether the isolates from India belonged to any of the serogroups in the panel. The panel consisted of the following representative serogroups:

- Australis
- Autumnalis
- Ballum
- Bataviae
- Canicola
- Celledoni
- Cynopteri
- Djasiman
- Grippopyphiosa
- Hebdomadis
- Icterohaemorrhagiae
- Javanica
- Mini
- Panama
- Patoc
- Ptomona
- Pyrogenes
- Sejroe
- Shimeri.

The isolates from India were subjected to virulence studies at the Departimento Di Scienze Biomediche, Trieste, Italy. This test was performed by exposing the isolates to 10% normal, fresh (complement intact) and heated (complement depleted) human serum.

**Results**

Out of a total of 1,348 paired kidney samples cultured, 296 samples were culture positive for spirochaetes (22%). Of the 120 isolates referred to the WHO/FAO Reference Laboratory in Brisbane, fifty-six isolates were identified. This includes twenty-three isolates of *Leptospira inadai* (including one isolate from a healthy rabbit), and thirty-three *Leptonema* isolates. The remaining sixty-four isolates have not yet been classified. The highest number of *L. inadai* isolates (ten
isolates) was obtained from *R. hinton*, followed by *B. bengalensis* (five isolates), *R. rufescens* (four isolates) and *B. indica* (three isolates).

*Leptospira inadai* and *Leptonema* were never isolated from the same rodent (Tables I and II). Preliminary tests to ascertain whether the isolates were free-living saprophytic leptospires revealed that the organisms grew in media containing 225 µg/ml of 8-azaguanine, and at 30°C, but did not grow at 13°C. Most cells were converted into spherical forms in media containing 1 M sodium chloride.

The results of the serum killing test performed at the Departimento Di Scienze Biomediche, Italy, revealed that *L. inadai* were moderately or totally serum resistant (M. Cinco, personal communication).

Preliminary MAT results suggested that the isolates from India had a variable reactivity against serogroups Javanica, Ballum, Pyrogenes, Pomona, Djasiman, Mini, Shermani, Patoc I, Celledoni and Sejroe (21).

The molecular studies performed at the Leptospirosis Reference Laboratory at Brisbane established that the RAPD profiles of the isolates from India were different from those of the contaminating strains of *L. inadai* available at the reference laboratory. Amplification of the 16S-23S rDNA spacer of the isolates from India was negative.

### Discussion

*Leptospira inadai* was first isolated from a man suffering from Lyme disease in 1981, although the cause of disease was later found to be *Borrelia burgdorferi*. It is now accepted that *L. inadai* was only a concurrent infection in this individual. To the knowledge of the authors, no record exists of isolation of *L. inadai* from any host other than this single human case.

Further studies on the *Leptonema* isolates from India are under progress, and it would be premature to form any opinion on the possible significance of these isolations.

The prominent feature of this study is the recognition, for the first time, that *R. hinton*, *R. rufescens*, *B. indica* and *B. bengalensis* constitute the rodent fauna harbouring *L. inadai*. These rodent species are widespread throughout India (Zoological Survey of India, Calcutta, personal communication) and information on the leptospiral carrier status of these species has not been established in India.

Several serovars of *Leptospira*, such as *Icterohaemorrhagiae*, *Canicola*, *Grippotyphosa*, *Pyrogenes*, *Hebdomadis*, *Australis* and *Javanica*, have been isolated from various hosts by workers in India, and the present isolation of *L. inadai* adds to the multiplicity of leptospiral species prevalent in the country. The preponderance of *L. inadai* in four different species of rodents and the absence of any other *Leptospira* suggest that these animals could be the natural reservoir hosts of this bacterium. However, these findings will need support from further field and laboratory studies before the pathogenicity and reservoir status of the isolates of this particular species of *Leptospira* can be confirmed in humans or other animals.

### Table I

**Rodent habitats and isolation of leptospires**

<table>
<thead>
<tr>
<th>Habitat</th>
<th><em>Rattus hinton</em></th>
<th><em>Rattus rufescens</em></th>
<th><em>Bandicota indica</em></th>
<th><em>Bandicota bengalensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of samples</td>
<td>Number of isolations</td>
<td>Number of samples</td>
<td>Number of isolations</td>
</tr>
<tr>
<td>Dairy farms</td>
<td>396</td>
<td>86</td>
<td>186</td>
<td>49</td>
</tr>
<tr>
<td>Human dwellings</td>
<td>303</td>
<td>71</td>
<td>171</td>
<td>21</td>
</tr>
<tr>
<td>Laboratory animal house</td>
<td>49</td>
<td>21</td>
<td>29</td>
<td>8</td>
</tr>
<tr>
<td>Animal feed plant</td>
<td>48</td>
<td>4</td>
<td>21</td>
<td>3</td>
</tr>
<tr>
<td>Kennel</td>
<td>18</td>
<td>1</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>819</td>
<td>183</td>
<td>412</td>
<td>81</td>
</tr>
</tbody>
</table>

**Isolation rate:** 22%

### Table II

**Isolation of *Leptospira inadai* from different rodent species**

<table>
<thead>
<tr>
<th>Rodent species</th>
<th>Habitat</th>
<th><em>Rattus hinton</em></th>
<th><em>Rattus rufescens</em></th>
<th><em>Bandicota indica</em></th>
<th><em>Bandicota bengalensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of samples</td>
<td>Number of isolations</td>
<td>Number of isolations</td>
<td>Number of isolations</td>
<td>Number of isolations</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dairy</td>
<td>6</td>
<td>3</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Human dwellings</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Laboratory animal house</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Animal feed plant</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kennel</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Isolation rate:** 22%
A large number of isolations from the rodent population of a particular geographical area is intriguing and raises the question of whether these strains are dominant over other strains of leptospires. However, a complete picture of the serovar distribution in this region will be available only after all the typing results are available. This has revealed a new host-pathogen/parasite relationship and raises a critical question as to the likely impact of this relationship on the renal carrier status of other Leptospira pathogenic to man and animals in this part of India.

Leptospira inadai is believed to occur as a common contaminant in cultures. The authors have therefore critically analysed this possibility. Commercial ready-to-use EMJH supplements, which are often incriminated as the source of contaminants, are never used in this laboratory. The EMJH supplement was always freshly prepared with BSA fraction V. An analysis of the results of the identification of the leptospires, has revealed that consecutive cultures yielded different isolates, not uniformly of any one type. Furthermore, the results of the RAPD clearly indicated that the isolates from India had a distinctly different genomic sequence compared to the contaminant strains maintained at the WHO Centre for Reference and Research on Leptospirosis, in Brisbane (21). Therefore, contamination of the isolates of L. inadai with saprophytic free-living leptospires was not possible.

On the basis of physiology, morphology, pattern of flagellar insertion, fatty acid extracts of methyl ester, guanine + cytosine ratio and DNA analysis by PCR, L. inadai has been classified as a member of the pathogenic group (1, 7, 8, 9, 15, 22). Furthermore, L. inadai may represent an emerging transitory phylogenic subline, since the species possesses both pathogenic and saprophytic domains (9, 21).

Experimental pathogenicity studies have revealed that L inadai is able to establish infection in African green monkeys, spider monkeys, guinea-pigs and hamsters (15). The ability of these organisms to grow at 30°C and to convert into spherical forms in media containing 1 M sodium chloride, together with the findings of serum killing tests performed by Dr M. Cinco, indicate that the strains were pathogenic. Complement resistance is an attribute of pathogenicity of leptospires. The isolates of L. inadai from India grew in a medium containing 8-azaguanine, a property attributed to the saprophytic leptospires only; parasitic strains do not grow in the presence of 8-azaguanine (4).

The growth behaviour of L. inadai in medium containing 8-azaguanine is rather intriguing. Parasitic strains which responded ambiguously to the tests to establish pathogenicity have been cited by earlier workers (12). The prevailing opinion is that L. inadai is probably under transition from saprophytic to pathogenic form (9, 21), which may explain the unusual response of L. inadai in the presence of 8-azaguanine. However, further studies are required to confirm the pathogenic status of L. inadai.

It is noteworthy that one of the L. inadai isolates was obtained from the blood of an apparently healthy rabbit from a small animal house where two isolations of L. inadai were made from R. hinton. Isolation from the rabbits was attempted only as part of a routine screening of the laboratory animals. The evidence does not allow any conclusion as to whether infection of the healthy rabbit and of R. hinton, which had free access to the laboratory animal house, was due to cross-infection between the two species of animals or a mere coincidence. However, no history or evidence of leptospiral infection is reported in the small animal house or the other locations in which rodents were trapped for isolation studies. In view of the present findings, future serological studies should include the local isolates of L. inadai in the battery of leptospiral antigens to establish the seroprevalence of L. inadai in the population.

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The authors thank Professor M. Cinco of Departimento Di Scienze Biomediche, Trieste, Italy, for conducting the complement inhibition tests, Dr Kiransingh, Deputy Director General (Animal Science) and Dr Lalkrishna, Assistant Director General (Animal Health), Indian Council of Agricultural Research, New Delhi, for encouragement, and the Director, Zoological Survey of India, Calcutta, for identifying the rodents.
Réservoirs de *Leptospira inadai* en Inde

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Résumé

Des études sur la virulence, menées à l’Université de Trieste en Italie, ont révélé que des isolats de *L. inadai* en provenance d’Inde étaient légèrement, sinon totalement, résistants au sérum lorsqu’ils étaient soumis au test de survie en présence du sérum. Pour établir la séroprévalence possible de cette espèce dans la population, il est recommandé d’inclure *L. inadai* dans la batterie d’antigènes utilisés pour les besoins d’études séro-épidémiologiques sur la leptospirose.

Mots-clés

Reservorios de *Leptospira inadai* en la India

N.L. Gangadhar, M. Rajasekhar, L.D. Smythe, M.A. Norris, M.L. Symonds & M.F. Dohnt

Resumen
Los autores describen la experiencia realizada en Bangalore (sur de la India) para intentar aislar *Leptospira* en tejido renal de *Rattus rattus wroughtoni hinton*, *Rattus rattus rufescens*, *Bandicota bengalensis* y *Bandicota indica*. A partir de un total de 1 348 cultivos de riñón, se obtuvieron 296 espiroquetas (lo que representa una tasa de aislamiento del 22%). Utilizando técnicas serológicas y de amplificación en cadena por la polimerasa (PCR) pudo identificarse un lote de cincuenta y seis de los organismos aislados en la India. El Centro Colaborador de Investigación y Referencia sobre la Leptospirosis (Brisbane) de la Organización Mundial de la Salud/Organización de las Naciones Unidas para la Agricultura y la Alimentación determinó que veintitrés de esas cepas correspondían a *L. inadai*. Se trata del primer caso registrado de aislamiento de *L. inadai* en roedores. La predominancia de este microorganismo en cuatro especies distintas de roedores sugiere que tal vez estos animales constituyan su reservorio natural, y abre un interrogante fundamental acerca de la probable relación entre la presencia de...
esta especie y la existencia de portadores renales de otras *Leptospira* patógenas para el hombre y los animales en esta región de la India. Los estudios de virulencia realizados en la Universidad de Trieste (Italia) pusieron de manifiesto que los cultivos de *L. inadai* aislados en la India presentaban una resistencia entre moderada y total a la prueba de supervivencia en presencia de suero (*serum killing test*). A fin de determinar la posible seroprevalencia de esta especie en la población, los autores recomiendan que se incluya *L. inadai* en la batería de antígenos leptospirosales utilizados en los estudios seroepidemiológicos.

**Palabras clave**

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**References**


