

Original Article

Human Leptospirosis in Erode, South India: Serology, Isolation, and Characterization of the Isolates by Randomly Amplified Polymorphic DNA (RAPD) Fingerprinting

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SUMMARY: The study describes the first attempt to record leptospirosis in Erode by isolation and serological tests such as the microscopic agglutination test and IgM-based enzyme-linked immunosorbent assay. Twenty-nine clinically suspected cases showing fever, headache, body ache associated with jaundice, decreased urine output, and conjunctival suffusion were included. The age of the patients ranged between 10-71 years and most of them were agricultural workers. Paired sera were possible among 12 cases. All the patients had fever and headache and other more common symptoms were myalgia and icterus. Leptospiral culture was positive in 7 (24.1%) patients. Out of 29 patients, 26 (89.7%) were diagnosed as having current leptospiral infection based on serology and isolation. The leptospiral isolates KSR 1-6 were further characterized by using randomly amplified polymorphic DNA fingerprinting shown genetic similarities with *Leptospira interrogans* spp. This study shows the presence of leptospirosis among the hospital cases of Erode and that this disease is a potential health hazard of agricultural workers in Cauvery basin.

INTRODUCTION

Leptospirosis is known to be endemic in Chennai city and several other places in the southern state of Tamilnadu in India (1,2). Erode is a town on the banks of the Cauvery River in the mid-western part of Tamilnadu (Fig. 1). The town is surrounded by rice fields and small rural communities. Erode has a tropical climate throughout the year with temperatures ranging from 24 to 32°C and receives 1,200-1,800 mm rainfall annually. Most of the rainfall is during the monsoon season in the months of October-December. This climatic condition is ideal for the survival of leptospire, particularly during the monsoons when the rice fields are mostly flooded or wet. Farming practices are by and large conventional and the farmers go barefoot in the waterlogged fields for sowing, weeding, and harvesting. Because of these factors, leptospirosis is a potential health problem of the rural communities located on the Cauvery riverbank. In Chennai city, leptospirosis accounts for about 30% of the cases of pyrexia of unknown origin (PUO) during the monsoon season. It is also the major cause of renal failure (3). Although Erode and the surrounding villages have ideal epidemiological conditions for the transmission of leptospirosis, no study on leptospirosis has been conducted in this area. We conducted a hospital-based study on the presence of leptospiral infection among patients with symptoms/signs suggestive of leptospirosis during the monsoon season in 2000. The cases were confirmed by serology and isolation, and the isolates were characterized

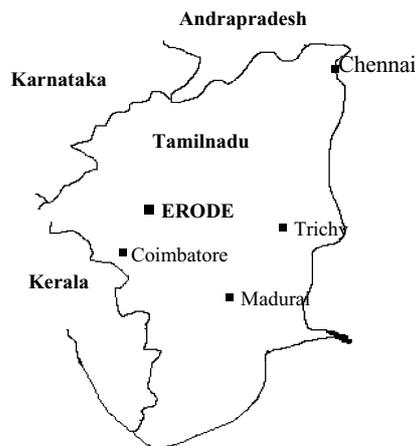


Fig. 1. Map of State Tamilnadu, South India.

by means of randomly amplified polymorphic DNA (RAPD) fingerprinting.

MATERIALS AND METHODS

This study included patients admitted to Government General Hospital, Erode, which draws most of the patients from the town of Erode and surrounding villages. The inclusion criteria consisted of the presence of fever with headache or body aches associated with (a) jaundice, (b) decreased urine output, or (c) bleeding tendencies including subconjunctival hemorrhage. Blood and urine samples were collected from the patients on the day of admission. Attempts were made to collect a second sample 7-10 days after collection of the first sample. Serum was separated from all the blood samples and

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stored at -70°C until use.

Microscopic agglutination test (MAT) was performed on the samples using nine live leptospiral strains as antigens. The strains belonged to the serogroups Australis (strain Jez-Bratislava), Autumnalis (Akiyami A), Ballum (Mus 127), Bataviae (Swart), Canicola (Hond Utrecht IV), Icterohaemorrhagiae (RGA), Grippotyphosa (Moskva V), Hebdomadis (Hebdomadis), and Pomona (Pomona). MAT was done at doubling dilutions starting from 1:20. Positive samples were titrated up to end titers. A titer of 1:80 or above was considered positive. It has been shown that a titer of 1:50 is the ideal cut-off titer in non-endemic areas and a titer of 1:200 is the ideal cut-off in highly endemic areas (4). Since no information about seroprevalence in Erode was known, a titer in between these two titers was considered the cut-off titer.

IgM ELISA was performed using ELISA plates prepared in-house. The plates were coated with heat-killed whole cell antigen prepared from Patoc I strain and the ELISA test was done according to the standard procedure (5). A titer of 1:80 is considered indicative of current leptospiral infection.

Isolation of leptospires was attempted from the blood and urine samples of the patients. Blood samples were inoculated into EMJH semisolid medium (6). Urine samples were inoculated into EMJH semisolid medium containing 2% rabbit serum and 100 $\mu\text{g/ml}$ of 5-fluorouracil (7). The cultures were examined every 10 days for up to 6 months. Positive samples were sub-cultured into fresh EMJH semisolid media.

Serotyping of the isolates were performed by MAT using group sera according to the standard procedure (8) using a panel of 36 group-specific rabbit antisera (group sera) representing all pathogenic serogroups. An isolate was considered to belong to the serogroup of the group serum that gave the highest titer (9). Serovar level identification was done by cross-agglutination test (CAT) using MAT against all reference antisera of the identified serogroup in order to establish cross-reactivity patterns. CAT was performed following a standard procedure (9) to identify the serovar status of the isolates. The serotyping of the isolates up to serovar level was carried out at the WHO/FAO Collaborating Center for Reference and Research on Leptospirosis, Brisbane, Australia.

Patients fulfilling any of the following criteria were considered cases of leptospirosis: i) Positive isolation of leptospires from blood or urine, ii) Sero-conversion or four-fold rise in titer in MAT for those with paired samples, iii) A titer of 1:80 or more with a positive IgM ELISA (titer of 1:80). The infecting serogroup was determined based on the results of serotyping in the case of patients showing positive cultures and based on the highest MAT titer in the case of culture-negative patients.

DNA was prepared as per Boom et al. (10) from the leptospiral strains representing eight genomospecies viz., RGA, Ballico (*L. interrogans*); Wumalasena, 3522C, Kipod-179 (*L. kirschneri*); Perepelitsin, Sari (*L. borgpetersenii*); L-60T (*L. alexanderi*); CZ188, 1342-K (*L. santarosai*); Celledoni (*L. weilii*); CZ-214K (*L. noguchii*); ICF (*L. meyeri*) and from the isolates KSR1-6.

RAPD fingerprinting was performed according to the method described by Ramadass et al. (11). The reaction was carried out in a total volume of 50 μl consisting of 50 ng of leptospiral chromosomal DNA, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 4 mM MgCl_2 , each of the four deoxynucleoside triphosphates at a concentration of 0.1 mM, each primer at a

concentration of 300 μM , and 0.5 U of *Taq* DNA polymerase. PCR was carried out in a DNA Engine (MJ Research PTC 200) thermal cycler. The first 2 cycles consisted of a denaturation at 95°C for 5 min, annealing of primers for 5 min at 40°C , and extension for 5 min at 72°C . The subsequent 35 cycles consisted of denaturation for 1 min at 95°C , annealing of primers for 1 min at 60°C , and extension for 3 min at 72°C , with a final extension step for 10 min during the last cycle. The PCR products were analysed by electrophoresis on a 1% agarose gel at 70V for 3 to 4 h, and the RAPD profiles were documented by using a gel documentation system (Bio-Rad, Hercules, Calif., USA) and analysed by using Quantity 1-D analysis software, and the dendograms were formed with 4.0% tolerance in UPGAMA. The primers used were primers B11 (CCGGAAGAAGGGGCGCCAT) and B12 (CGATTTA GAAGGACTTGCACAC).

RESULTS

During the period of October-December 2000, 29 cases suspected of having leptospirosis based on the clinical criteria described above were admitted to the Government General Hospital, Erode. One of the patients died of renal failure to produce a case fatality rate of 3.4%. The age of the patients ranged between 10-70 years and the median age was 42.5 years. Twenty-seven (93.1%) of the 29 patients were males. All the patients except one were agricultural laborers. All the patients had fever and headache. The next common symptom was myalgia followed by icterus. None had severe bleeding tendencies. The prevalence of various symptoms/signs is summarized in Table 1. Acute blood samples were collected from all the patients. Follow-up samples could be obtained in the case of 12 patients.

Culture was positive in 7 (24.1%) patients. Both blood and urine samples were positive in one patient. In 3 patients blood samples were positive and in another 3 urine samples were positive. Out of the 8 isolates (from 7 patients) 2 were lost. Among the remaining, one isolate from the blood of a patient was typed as serovar Canicola, 2 were serovar Icterohaemorrhagiae and 3 were of serovar Australis based on serotyping. Primers B11 and B12 produced characteristic band patterns in RAPD analysis in the range of 2000 and 200 bp (Fig. 2). In comparison with the 8 genomospecies utilized, the isolates KSR 1-6 showed a close relationship with *L. interrogans* spp. by cluster into the same clone (Fig. 3).

Among the 12 patients who had paired samples, 2 showed a fourfold rise in titer and none of the other patients showed any signs of seroconversion except one who had some titers

Table 1. Common symptom/sign observed among the patients

Symptom/sign	%
Fever	100
Headache	100
Myalgia	83
Pyrexia of unknown origin	38
Conjunctival suffusion	31
Nausea	31
Jaundice	28
Vomiting	21
Renal failure	7
Splenomegaly	7

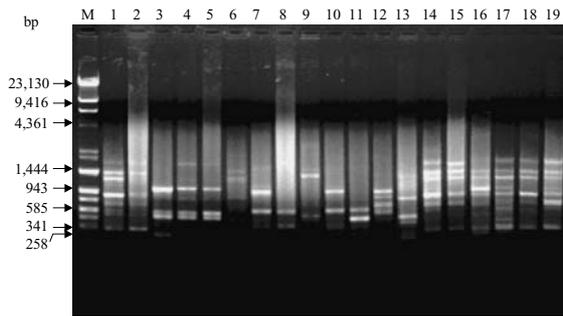


Fig. 2. RAPD analysis of leptospiral isolates in comparison with leptospiral strains from different genomospecies. Lane particulars: M, λ DNA/Hind III; 1, RGA; (Icterohaemorrhagiae); 2, Ballico (Australis); 3, Wumalasena (Grippotyphosa); 4, 3522C (Cynopteri); 5, Kipod-179 (Grippotyphosa); 6, Perepelitsin (Tarassovi); 7, Sari (Mini); 8, L60T (Manhao); 9, CZ188 (Grippotyphosa); 10, 1342-K (Shermani); 11, Celledoni (Celledoni); 12, CZ-214K (Panama); 13, ICF (Ranarum); 14, KSR1 (Icterohaemorrhagiae); 15, KSR3 (Icterohaemorrhagiae); 16, KSR4 (Canicola); 17, KSR5 (Australis); 18, KSR6 (Australis); 19, KSR2 (Australis).

in the first sample itself and one whose first sample was negative and did not show any titer in the second sample. However, this MAT-negative patient had a positive IgM ELISA test. One patient had a titer of 1:40 in the first sample and 1:80 in the second sample. All others had titers above 1:160 in both the samples. Thus, 11 out of the 12 patients with paired samples fulfilled the MAT diagnostic criteria. All of them showed positive IgM ELISA as well, hence these 11 patients fulfilled the criteria for diagnosis of leptospirosis. Three among these 11 patients also showed positive isolation.

Among the 17 patients who had only a single sample, 2 were negative. These 2 did not yield positive cultures either. One had a positive IgM ELISA result. Fifteen patients had MAT titers of 1:80 or above and all of them showed positive IgM ELISA as well. Thus these 15 patients fulfilled the diagnostic criteria. Among these 15, 9 had a titer of 1:80 and the remaining 6 had titers in the range of 1:160 to 1:10240. Four among these 15 showed positive isolation.

Twenty-six (89.7%) of the 29 patients were thus diagnosed as having current leptospiral infection (11 among those with paired samples and 15 among those with a single sample).

Table 2. Distribution of antibody titers as determined by MAT and IgM ELISA

Titer	MAT (+ve 26/29)		IgM ELISA (+ve 27/29)	
	Number	%	Number	%
1:80	10	34.5	9	31.0
1:160	4	13.8	2	6.0
1:320	4	13.8	3	10.3
1:640	1	3.4	3	10.3
1:1280	2	6.8	2	6.9
1:2560	–	–	1	3.4
1:5120	4	13.8	5	17.2
1:10240	1	3.4	1	3.4
1:20480	–	–	1	3.4
Total	26	89.6	27	93.1

The distribution of MAT titer among these 26 patients is shown in Table 2. Ten had a titer of 1:80 and the remaining 16 had titers in the range of 1:160 to 1:10240. Presumptive information about the infecting serogroup based on highest MAT titers could be obtained in 23 of the 26 patients. The most common was Australis, which was seen in 9 (34.6%) followed by Hebdomadis in 4 (15.4%), Grippotyphosa and Icterohaemorrhagiae was seen in 3 each (11.5%), and Autumnalis and Canicola in 2 each (7.7%). In the remaining 3 patients MAT showed equal titers against more than one serovar.

Out of the 29 patients, 26 were positive by MAT and 27 by IgM ELISA. Compared to MAT, ELISA had a sensitivity of 96.3% (79.1-99.8), a specificity of 100% (19.8-100), a positive predictive value of 100% (84.0-100), and a negative predictive value of 66.7% (12.5-98.8). The overall agreement between the tests was 96.3% and the kappa value of agreement was 0.7820 ($Z = 4.31, P < 0.001$). The clinical criteria used for diagnosis had a predictive value of 89.7% (26/29).

DISCUSSION

The study shows that leptospirosis occurs frequently in Erode and surrounding villages on the Cauvery basin during the north-east monsoon. Considering the environmental conditions and occupational habits of the population, this is

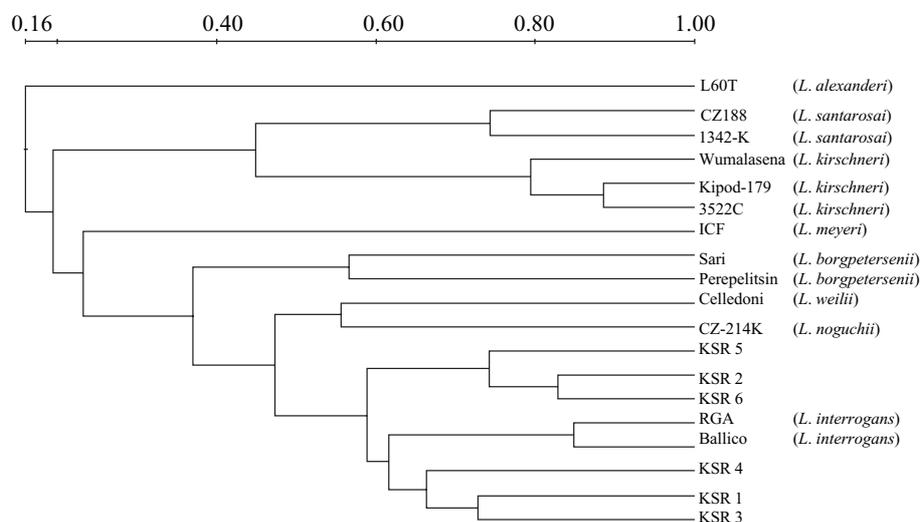


Fig. 3. Phylogenetic tree for the isolates KSR 1-6 in comparison with different genomospecies.

expected. However, in the absence of studies, the presence of leptospirosis has never been detected, and hence the clinicians were not alert to the possible presence of leptospirosis while diagnosing cases of febrile illness.

The clinical criteria used for screening patients had a strong predictive value. However, there is a possibility that its sensitivity is low. This can only be tested by screening samples of all fever cases for leptospirosis and following them up for the development of symptoms/signs suggestive of leptospirosis. As it is known that in most cases leptospirosis presents as a mild flu-like illness, the actual number of leptospirosis cases could be several-fold the number estimated by screening the patients clinically diagnosed as having leptospirosis.

There is a possibility that the cut-off titer used for MAT (1:80) may not be appropriate as there is no information about the background seroprevalence. However, in the present study, patients with only a single sample were diagnosed to have leptospirosis only when they showed positive results in both MAT and IgM ELISA. A positive IgM ELISA indicates that the infection is current or in the recent past and the MAT titer is not due to past infection. Hence the diagnostic criterion is expected to have a fairly high degree of specificity.

Other than non-specific symptoms/signs such as fever, headache and myalgia, jaundice was the most common symptom presented. Renal failure was seen in two patients, one of which died. Pulmonary complications that are commonly seen in some places in India (12) were not observed. This could be because the clinical criteria did not include these conditions. None of the patients had nervous system complications such as convulsion or meningitis.

The serogroup Autumnalis is the dominant one in most places in Tamilnadu. In contrast, in the present study, Australis was the dominant one. Until now, the serogroup Australis has not been isolated from human cases in Tamilnadu. In the present study, three isolates belonging to serogroup Australis were obtained. The epidemiological settings of earlier studies were different from the present one. Earlier studies were either in cities (2,3), where the exposure was to overflowing sewage and stagnant rainwater on the roads or to wet surrounding rice mills (13). In the villages surrounding Erode, the major exposure is to stagnant water in rice fields. It is possible that the transmission dynamics are different here and hence the dominant serogroup is also different.

IgM ELISA prepared in-house showed high degrees of sensitivity and specificity in comparison to MAT. However, the number of samples was less in order to make accurate estimates of indices of accuracy. The confidence intervals were wide in the case of specificity and negative predictive values.

The molecular characterization of the isolates indicates that RAPD fingerprinting is one of the simpler methods for characterizing the isolates on the species level (11). This method produced bands that were distinct, reproducible, and species-specific. Within the species, this method also showed some discrimination between the serovar level as has been reported earlier (14). In the present study leptospiral reference strains from eight genomospecies were included for comparison and the isolates KSR 1-6 had similarities with *L. interrogans* spp.

The study shows that leptospirosis is a potential health hazard of rice field workers in Cauvery basin. Leptospirosis has already been included in the proposed integrated disease surveillance program as a disease condition of local interest. The exact situation of leptospirosis in different parts of the

state will be clear once the surveillance system starts operating.

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