Leptospiral renal colonization status in asymptomatic rural population of Tiruchirapalli district, Tamilnadu, India

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Humans are known to excrete leptospires in their urine after recovery from illness and there are reports showing development of asymptomatic leptospiuria in settings of high disease transmission. In this regard, we sought to evaluate the asymptomatic renal carriage status of humans in the highly endemic region of Tiruchirapalli district, Tamilnadu, India. A total of 245 asymptomatic participants were included. Urine and blood samples were collected and the extent of leptospiral infection was characterized by MAT, qPCR, 16S rRNA, and dot blot assay. The qPCR screening with urine DNA identified 129 (52.7%) positive samples further confirmed by nested PCR. The dot blot assay marked 30.2% (74/245) as true positives. The phylogenetic analysis showed the sequences to cluster with pathogenic *Leptospira* spp. Serological results showed 50 people with urine positivity to be negative for MAT and can probably be classified as 'asymptomatic individuals.' In conclusion, it can be speculated that in endemic regions there is a greater possibility of humans as maintenance host rather than incidental hosts.

Keywords: *Leptospira*, Leptospiuria, MAT, qPCR, Incidental hosts

Introduction

Leptospirosis is a zoonotic disease caused by pathogenic spirochetes of the genus *Leptospira*. Although leptospirosis has been reported worldwide it is more common in tropical and sub-tropical areas where environmental and socioeconomic conditions favor its transmission. The disease is principally transmitted to humans indirectly by contact with water or soil contaminated with urine of domestic and wild animals with persistent renal infection.^{1–3}

A large number of mammals serve as reservoir hosts for leptospires. The most frequent hosts are rodents, especially the common rat (*Rattus norvegicus*). In the reservoir host, the organisms persist indefinitely in the convoluted tubules of the kidney without causing apparent disease, and are shed in the urine in massive numbers.⁴ There are a few reports of humans as accidental asymptomatic carriers of *Leptospira*. Bovet *et al.*⁵ showed 9–48% of healthy subjects to have asymptomatic leptospiral infection by serology (ELISA-IgM) and PCR. Humans develop asymptomatic leptospiuria and shed pathogenic *L. interrogans*

and intermediate pathogens *L. licerasiae* in their urine.^{6,7} Asymptomatic leptospiral infection has been predicted to be common in endemic areas and has been reported in several studies.^{8,9}

India with its tropical climate and agriculture-related occupational activities seems to be an ideal niche for a high level of transmission of leptospirosis. The disease has been reported to be endemic in Kerala, Tamilnadu, Gujarat, Andamans, Karnataka, Maharashtra, Andhra Pradesh, Orissa, West Bengal, Uttar Pradesh, Delhi, and Pondicherry.^{10,11} Human leptospirosis was reported in Chennai, Tamilnadu for the first time in 1983.¹² After this preliminary study, there were no organized reports on the prevalence of human leptospirosis from Tamilnadu for more than two decades. A sudden increase in the prevalence of leptospirosis was reported between 2004 and 2006 in Chennai. In 2009, we observed an upsurge in the prevalence of leptospirosis in Tiruchirapalli district, Tamilnadu.¹³

There is paucity of information on prevalence of asymptomatic leptospiral infection and leptospiral renal carriage in endemic areas in India. In view of this, we carried out a population-based study to estimate the prevalence of leptospirosis among healthy individuals and their asymptomatic renal carriage status in Tiruchirapalli district, Tamilnadu, India.

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Materials and methods

Study site

The study was carried out in the village of Tiruvalarchipatti, a rural community located 18 km south of Tiruchirappalli, Tamilnadu, India. The village with its tropical climate (range 28–38 °C), geographical location of 10.81° N Latitude and 78.69° E Longitude and a total area of 4403.83 km² is surrounded by 18 tannery units. The village comprises of approximately 2000 residents living in about 400 households. Tannery work, agriculture, cattle rearing, poultry husbandry, and fisheries are their major occupations. The source of potable water for the village is from public tap as the open draining of effluent from tannery has rendered the ground water harmful. The village has open sewage and poor drainage system making the environment unhygienic. Farm animals (cattle, goat, sheep, dog, duck & pigs) move liberally throughout the village. Rat infestation is a common complaint of the inhabitants and the rats captured in this study area harbor pathogenic *Leptospira* species.¹⁴

Study subjects and sample collection

As a part of our program to assess the burden of leptospirosis and develop prevention strategy, collaboration with the local health centers and government hospitals were set up. The public health nurses visited the households, educated the people about the risk factors, preventive and precautionary steps for leptospirosis risk reduction and invited the participation of the people in the study. The purpose and procedures of the study were explained to study subjects and informed written consent was obtained from participants or parents/guardians (for participants ≤17 years of age). Subjects were excluded if they had fever within the previous 2 weeks or if they declined participation (Fig. 1). All participants were clinically evaluated and interviewed for the socioeconomic status and behavioral pattern. Blood and urine samples were collected from the enrolled participants. This study was approved by Institutional ethics committee (IEC) of Bharathidasan University, Tiruchirappalli, India (Ref No.DM/2010/101/13).

Figure 1 Flowchart showing the design of the study and the process for the identification of asymptomatic human carriers.

A 30% prevalence for asymptomatic leptospiral carriage status was predicted (approximately the mid-point of the range reported previously by Bovet et al.⁵ To estimate this prevalence with a relative precision of 20% or an absolute precision of 6% and with 95% confidence limits, a sample size of 225 was required. Then it was decided to do an oversampling by approximately 30% to accommodate for non-participation and non-compliance with instructions. Thus the final sample size achieved was 290. Households were randomly selected and members of the household who volunteered to participate in the study were enrolled till the required number of subjects were enrolled.

Serological analysis

Venous blood samples (5 mL) were collected in tubes without anticoagulant and transported to laboratory on ice. Serum was separated, frozen in aliquots at -20°C until used. Microscopic agglutination test (MAT) was performed with a panel of 13 locally circulating leptospiral serovars.¹³ All the serovars were maintained with periodic subculture in EMJH medium at the Medical Microbiology Laboratory, Bharathidasan University, Tiruchirapalli. Seven days old culture at a concentration of $1-2 \times 10^8$ organisms/mL was used as antigen in MAT. Each serum was tested in doubling dilutions starting from 1:20. MAT titers were reported as the reciprocal of the dilution agglutinating $\geq 50\%$ of live bacterial antigen and a titer of ≥ 1 in 160 was considered positive.¹³

Analysis of urine samples for isolation of leptospires and DNA extraction

Urine samples were collected in 50-mL sterile falcon tubes, and a portion of the sample was processed for isolation of leptospires in EMJH medium¹² and remaining were transported to the laboratory within 2 h for DNA extraction. The samples were pelleted at $3000 \times g$ for 30 min at room temperature. DNA was extracted from the pellet as described earlier with minor modifications.¹⁵ The extracted DNA was stored at -20°C until further analysis. The serogroup level typing of isolated leptospires was performed at WHO Reference Laboratory for Leptospirosis, Port Blair.

Quantitative real time PCR (qPCR) screening

The qPCR assay was performed using the CFX96 real-time system (Bio-Rad, Hercules, California, USA) using TaqMan assay targeting the 16S rRNA leptospiral gene as described previously.^{7,16}

Nested PCR amplification

Nested PCR for the identification of pathogenic leptospiral DNA in urine samples was achieved as described earlier.¹⁷ For each sample PCR was carried out at least three times by two different individuals for reproducibility in a thermal cycler (Eppendorf, Germany). PCR products were analyzed by electrophoresis on a 1.5% agarose gel and

stained for 20 min with 0.5 g/mL of ethidium bromide. Gel images were documented in a gel documentation system (Bio-Rad, Hercules, California, USA).

16S rRNA gene sequencing and analysis

The amplified fragments were gel extracted using QIAquick gel extraction kit (Qiagen, USA) as per manufacturer's instruction. The purified products were sequenced commercially (Macrogen, South Korea). The 16S rRNA gene sequences thus obtained were compared with other leptospiral sequences by using NCBI BLASTn for their pairwise identities. Multiple alignments of these sequences were carried out by Clustal W 1.83 version of EBI (<http://www.ebi.ac.uk/cgibin/clustalw/>) with 0.5 transition weight. Phylogenetic trees were constructed in MEGA 5.0 version (<http://www.megasoftware.net>) using unweighted pair group method with arithmetic mean (UPGMA) algorithms. UPGMA bootstrapping was performed with 1000 replicates with pseudorandom number generators. These sequences have been deposited in GenBank.

Dot-Blot analysis

Briefly, a 150-bp Biotin-labeled DNA probe was synthesized by PCR-labeling with the Biotin DecaLabel™ DNA labeling kit (Thermoscientific, USA). *Leptospira interrogans* serovar Autumnalis strain N2 genomic DNA and 16SF-5'CAAGTCAAGCGGAGTAGCAA3' 16SR-5'TAACCTGCTCTGCCTCCCGTA-3' was used as template and primer for probe synthesis. Genomic DNA from urine samples was PCR-amplified using the aforementioned nested PCR assay, and the PCR products were gel extracted using QIAquick gel extraction kit (Qiagen, USA). The purified PCR products were then denatured by boiling for 10 min in 20 X SSC buffer and then cooled on ice and spotted onto NC Membrane (Millipore, USA). After spotting, the membrane was air-dried and the DNA was fixed by UV cross-linking. The spotted membrane was hybridized overnight at 56°C with 5 pmol of the synthesized DNA probe and hybridized according to manufacturer's directions (Thermoscientific, USA). After hybridization and stringency washes, the blot was developed using the streptavidin-AP conjugate with BCIP/NBT as substrate.

Statistical analysis

The statistical analyses were performed using prism software (version 5.0c; Graph pad software, San Diego, CA) and Epi Info ver 6.0 (CDC, Atlanta, USA). Statistical significance of difference between means was determined by one-way analysis of variance (ANOVA) at a p value of ≤ 0.05 . Differences in proportions were tested by χ^2 test. Unmatched univariate analysis was performed to assess the association of behavioral factors and contact with animals to urine leptospiral positivity. Odds ratios were calculated with 95% confidence intervals.

Results

Study population

The study enrolled a random sample of 290 participants aged ≥ 5 years from the residents of 150 households picked from Tiruvallarchipatti. Of the 290, 45 participants were excluded as 16 reported febrile illnesses within 2 weeks of sampling and no urine samples available for 29 participants making a total of 245 participants. Of the 245, 98 (40%) were men and 147 (60%) were women. The median age of the study participants was 45.4 (range 18–65) with women being slightly (median age 47) older than men (median age 46.2) and did not differ significantly ($p > 0.05$).

Seroprevalence of leptospiral infection

Of the 245 participants, blood samples were available from 196 (80%). Of these, 66 and 130 were from males and females, respectively. Circulating anti-leptospiral antibodies were detected by MAT in 59 (30.1%, 95% CI: 23.8, 37.0) of the 196 subjects. The most frequently observed circulating serovars were Ballum (31/196, 16%) followed by Australis (20/196, 10%) and Autumnalis (8/196, 4%) (Table 1).

Isolation of leptospires from urine samples

In total, 13 isolates (5.1%) were obtained from urine samples of 245 participants. Among the 13 isolated, three isolates were obtained from subjects considered as asymptomatic urinary shedders. Among the 13 isolates 4 were lost in subsequent subcultures. Remaining 9 isolates were identified as serogroup Ballum (5), Australis (3) and Autumnalis (1). The three isolates from asymptomatic urinary shedders belong to Australis (2) and Autumnalis (1). The 16S rRNA sequence analysis of the isolates showed homology with the genomospecies *Leptospira interrogans*. MAT was performed against the three isolated *Leptospira* as antigens and corresponding human sera were used. Interestingly, no agglutination was observed among homologous sera and also no cross-reactivity was observed for heterologous sera which further confirmed that the isolates obtained were from asymptomatic urinary shedders. Future studies are required to genotype these isolates in order to characterize its local circulation among febrile patients.

Molecular identification of leptospiral species in urine samples

The initial qPCR screening identified 129 (52.7%) positive samples. Further evaluation of these samples with nested

Figure 2 Dot blot of the urine DNA samples of asymptomatic human carriers. 1–25 randomly used urine samples for this assay.

16S rRNA-based PCR confirmed the positivity in all the 129 qPCR positive samples. The dot blot assay with the probe designed in the present study marked 74 (57.4%) of the 129 qPCR positive samples, which is 30.2% (95% CI: 24.5, 36.4) of the 245 study subjects (Fig. 2). The 16S rRNA was sequenced for 10 representative dot blot positive samples and deposited in NCBI with accession numbers KJ676484, KJ650298, KJ658342, KJ676485, KJ676486, KJ683061, KJ676487, KJ676488, KJ676490, and KJ676491. Analysis of the sequences from dot blot-confirmed urine samples showed all the 10 representative gene sequences to cluster with pathogenic clades of *Leptospira* (Fig. 3).

Asymptomatic urinary shedding

Of the enrolled 245 inhabitants, 74 (30.2%) were confirmed to excrete *Leptospira* by detection of leptospiral DNA in their urine. Clinical, serological, and epidemiological data showed no evidence of recent infection in 50 of the 74 urine DNA positive subjects (20.4% of enrolled participants). These 50 subjects can be considered as ‘asymptomatic urinary shedders’ as they shed pathogenic *Leptospira* in their urine with no evidence of symptomatic or asymptomatic infection. Of the 50 participants with urine DNA positivity and serological negativity, 17 were men and 33 were women. The proportion of asymptomatic shedders among urinary excretors did not differ statistically between men and women ($p = 0.7$).

Serological characteristics of the participants with leptospiral DNA in urine

Serological results were available for 196 of the 245 participants including 69 of the 74 with *Leptospira* DNA positive urine. Fifty (27.5%) of these 69 subjects with urine positivity had negative results in MAT. The ability of MAT to correctly identify urinary shedders of *Leptospira*, which is its sensitivity, is only 27.5% (19/69, 95% CI:

Table 1 Seroprevalence of *Leptospira* among the study population

Serovar	MAT positives	
	% of positive	Median titer
Australis	33.3	1:1280
Autumnalis	6.6	1:1280
Ballum	53.3	1:640

Figure 3 Phylogenetic analysis of the 10 representative 16S rRNA sequences from urine DNA positive samples. Neighbor-joining method with 1000 bootstrap replication was used and NJ tree was constructed in MEGA6. Accession numbers of the 16S rRNA sequences used (KJ676484, KJ650298, KJ658342, KJ676485, KJ676486, KJ683061, KJ676487, KJ676488, KJ676490, and KJ676491).

17.8, 39.8). The overall agreement between urinary shedding of *Leptospira* and MAT was 54.1% with a κ statistic of -0.04095 indicating poor agreement.

Risk factors

The univariate analysis of the risk factors associated with urine positivity showed significant associations of having cattle in house, house in low-lying areas and exposure to stagnant water with leptospiuria (Table 2). Keeping dog in the house and rearing chicken were protective factors with low statistical significance.

Discussion

Leptospirosis is a relatively common zoonotic disease of tropical countries.¹⁸ The transmission of leptospirosis depends on several interacting factors. The important determinants include the presence and contact with reservoir animals, environmental conditions, and frequency of exposure to etiological agents, socioeconomic and lifestyle preferences of the individuals. In the present study, the handling of cattle, houses in low-lying areas and exposure to stagnant water were found to have a strong association

with leptospiral urinary shedding. It is likely that cattle act as the predominant carrier of *Leptospira* in the study community and a significant proportion of the population acquires infection from cattle directly or indirectly through the environment contaminated with cattle urine and then becomes asymptomatic urinary shedders of *Leptospira*. A high seropositivity (30%) was observed among the study individuals, who had no evidence of febrile illness during the preceding two months. Urine screening of asymptomatic individuals with PCR and dot blot-based analysis showed that at least 20% of the healthy population in the community shed leptospires in the urine without serological or clinical evidence of recent infection. Similar observation was made in healthy people from rural Amazonian community.⁷ They characterized 5% of the healthy population as urinary shedders of *Leptospira*.

It is generally accepted that humans can excrete leptospires from weeks to months after infection.^{19,20} Direct person-to-person transmission of leptospires is rare but possible.²¹ Other routes of transmission have been reported in a case where an infant was infected during breast feeding and a case of transmission during sexual intercourse.²²

Table 2 Risk factors associated with leptospiral urine DNA positivity

Variables	Urine DNA			
	Positive (N = 129)	Negative (N = 116)	OR (95% CI)	p Value
Dwelling near water bodies	36 (27.9)	15 (12.9)	2.61 (1.3, 5.1)	0.003941*
Low-lying location of house ^a	12 (9.3)	7 (6.0)	1.6 (0.61, 4.2)	0.6039
Use of public latrine	82 (63.6)	70 (60.3)	1.15 (0.68, 1.92)	0.3503
Mud floored house	4 (3.1)	6 (5.2)	0.59 (0.16, 2.13)	0.4132
Thatched roofed house	7 (5.4)	10 (8.6)	0.61 (0.22, 1.65)	0.3259
Public tap for drinking Water	108 (83.7)	96 (82.8)	1.07 (0.55, 2.10)	0.8403
Exposure to stagnant water	28 (21.7)	12 (10.3)	2.4 (1.16, 4.98)	0.0163*
<i>Animals</i>				
Cattle	51 (39.5)	15 (12.9)	4.4 (2.31, 8.41)	0.000003*
Goat	45 (34.9)	43 (37.1)	0.91 (0.54, 1.53)	0.7219
Dog	31 (24.0)	42 (36.2)	0.56 (0.32, 0.97)	0.0375*
Cat	24 (18.6)	21 (18.1)	1.03 (0.54, 1.98)	0.9194
Chicken	18 (14.0)	28 (24.1)	0.51 (0.26, 0.98)	0.0415*
No animal	38 (29.5)	43 (37.1)	0.71 (0.42, 1.21)	0.2591

^aLow lying location is an area located below the level of drainage system therefore water logged surrounding the houses after the rainfall and moist stay for extended periods of time.

* $p < 0.05$.

Humans are considered as incidental hosts and animals can be accidental or maintenance hosts. Since asymptomatic human carriers have been rarely documented, generally it is considered that asymptomatic infected individuals have an important role in disease maintenance and transmission.^{1,23–25} This probably is based on the perception that asymptomatic human urinary shedders of *Leptospira* are going to be rare in a community. In the wake of the present observations and a similar observation elsewhere, it may be necessary to re-examine this concept.

Leptospirosis has been reported to be endemic in all population groups of India. However the endemicity was proven by seroprevalence among occupational high-risk groups (range between 27 and 77%) and low-risk population group (15–20%).^{26–28} The interesting observation made through these studies was the presence of the same commonest serogroup among low- and high-risk population. This could likely be related to the presence of same circulating serovars, same transmission cycles of infection, and common environmental exposure risks.

The likelihood of asymptomatic carriers for leptospires may not be ruled out in this circumstances and prospective studies are required to examine this possibility. The present study provides evidence for shedding of leptospires in the urine by a significant proportion of the healthy individuals in an endemic community. The present study provided evidence of shedding of leptospiral DNA in the urine by a significant proportion of the healthy individuals in an endemic community. The ability of these shedders to play an important role in maintaining the transmission of leptospirosis depends on the presence of viable *Leptospira* in their urine. This is evidenced by ~6% by isolation and identification of the leptospires from the urinary shedders.

Another observation, which is of great importance, was the failure of microscopic agglutination test to correctly detect urinary shedders of *Leptospira*. Similar observations have been made in case of clinically suspected human leptospirosis cases or high-risk population groups²⁹

and as well as in cattle.³⁰ If human urinary shedders play a role in the maintenance of leptospiral transmission in endemic niches, the only possible method to identify them for intervention is going to be molecular tools as serology does not seem to help. Urine culture has low sensitivity and is time consuming, therefore suffers from the same drawback as MAT. This is going to pose an operational problem in leptospirosis endemic communities.

In conclusion, taking all these data together and from the previous report,⁷ it can be speculated that in endemic regions there is a greater possibility of humans being considered as asymptomatic urinary carriers, a feature attributed only to animals. Further exploring the similarity of the infecting strains across human and animal carriers in terms of causing infection can shed light on proposing humans as maintenance host rather than incidental hosts.

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