SEROPREVALENCE OF *LEPTOSPIRA BORGPETERSENII* SEROVAR JAVANICA INFECTION AMONG DAIRY CATTLE, RATS AND HUMANS IN THE CAUVERY RIVER VALLEY OF SOUTHERN INDIA

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Abstract. Leptospirosis is a major problem of dairy farms in Tamilnadu, India, resulting in abortions, stillbirths and infertility. Serologic and genetic analyses of samples from cattle, humans and rodents were performed in order to estimate infection prevalence and identify leptospiral species. Five hundred and fifteen sera and 76 urine samples were collected from dairy cattle on 25 farms including a farm that practiced rat control. Sera and kidney samples were also collected from field rats (*Rattus norvegicus*) in the vicinity of these farms. In addition, sera were collected from farm workers. Serum antibody was measured by the microscopic agglutination test. Leptospires isolated from blood, kidney, and urine were characterized as to serovar. Genomospecies were predicted using random amplified polymorphic DNA (RAPD) profiling. *SecY* gene sequencing was performed as a tool for tracing of source. Seroprevalence of 87.%, 51.% and 76.5% for cattle, rats and humans, respectively, was observed on endemic farms. Prevalences on a non-endemic farm were lower. Antibodies to Autumnalis, Javanica, Icterohaemorrhagiae and Pomona predominated in both cattle and rats. Thirteen isolates from rat kidneys were identified as serogroup Javanica, serovar Javanica. RAPD comparisons and *secY* gene sequencing identified these isolates as *Leptospira borgpetersenii*. These results altogether indicated that *L. borgpetersenii* was the dominant species in these areas with serovar Javanica apparently derived from rats which provided an important source of infection in cattle resulting a high incidence of infertility, abortion and still-birth in the Cauvery river valley, Tiruchirappalli, Tamilnadu.

Keywords: *Leptospira borgpetersenii* serovar Javanica, leptospirosis seroprevalence, cattle, humans, rodents

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INTRODUCTION

Infection of domestic animals with *Leptospira* can cause serious economic loss. Animals might serve as a reservoir of some serovars and become incidental

hosts of others. Incidental infections often result in severe or fatal disease in humans and dogs (Bolin, 2000). Dairy cattle have a role as a natural host of serovars Hardjo, Pomona, and Grippotyphosa, while pigs may harbor Pomona, Tarassovi, and Bratislava (Bolin, 2000). Sheep may harbor serovars Hardjo and Pomona, and dogs may harbor serovar Canicola (Bolin, 2000). Knowledge of the prevalent serovars and their maintenance hosts is essential to understand the epidemiology of leptospirosis. Domestic animals and humans acquire the disease from numerous feral species when their activities intrude into feral animal territories. Following infection, leptospiras localize in the kidneys and are intermittently shed in to the urine (Ellis and Michna, 1976). The local abundance of several species of pathogenic leptospiras may be a useful indicator of the potential for transmission of *Leptospira* spp to humans and livestock (Nascimento *et al*, 2004).

The Cauvery river valley in Southeast India has a heavy concentration of dairy farms that supply milk to the city of Tiruchirapalli. These farms experience recurrent economic losses due to infertility and abortion of the dairy cattle. Leptospirosis is most frequently diagnosed between September and November (Muthusethupathi *et al*, 1995) following the southwest monsoon season. Field rats (*Rattus norvegicus*) have been shown both to be carriers of *L. borgpetersenii* serovar Javanica in Tamil Nadu and have been implicated as a source of infection in sheep (Natarajaseenivasan and Ratnam, 1999, 2000).

The present study determined the prevalence of leptospira specific antibodies in sera of cows, rats and workers on dairy farms in the Cauvery valley and isolated and identified *Leptospira* spp in the kidneys of these rats.

MATERIALS AND METHODS

Surveillance site

Farm herds were situated in suburban areas of Tiruchirappalli, along the river Cauvery in central Tamil Nadu, Southeast India (Fig 1). Cows were grazed on commonage as a consolidated herd but were separated into small groups in the evening for milking and feeding at individual owner's dwellings. Local rainfall is mostly from the northeast monsoons (364.9 mm) and southwest monsoons (357.7 mm). Leptospirosis is most frequently found between September and November, with sporadic cases at other times. Cattle farms included for the investigation were operated as a group of organized herds with free access to rodents from surrounding fields. Cattle breeds were mainly Jersey, Sindhu and mixed breed. Rats had free access to grain-rich paddy hay, the major food of dairy cattle in Tamil Nadu and were captured by professional trappers in fields grazed by the cattle. Traps were set in the evening and captured rats were collected next morning. Control herd utilized a rodent trapping program and was located in an enclosure protected by a wall. Tamil Nadu dairy farm workers customarily work barefoot without protective covering or gloves and therefore are potentially exposed to *Leptospira* spp in aborted fetuses, membranes and urine from infected cows. Milking was done manually.

Samples

Five hundred and fifteen serum samples from dairy cows were collected on 25 farms located along the river Cauvery (Fig 1). Seventy-six urine samples were also collected from recently aborted cows. Blood (5 ml) was obtained from 51 farm

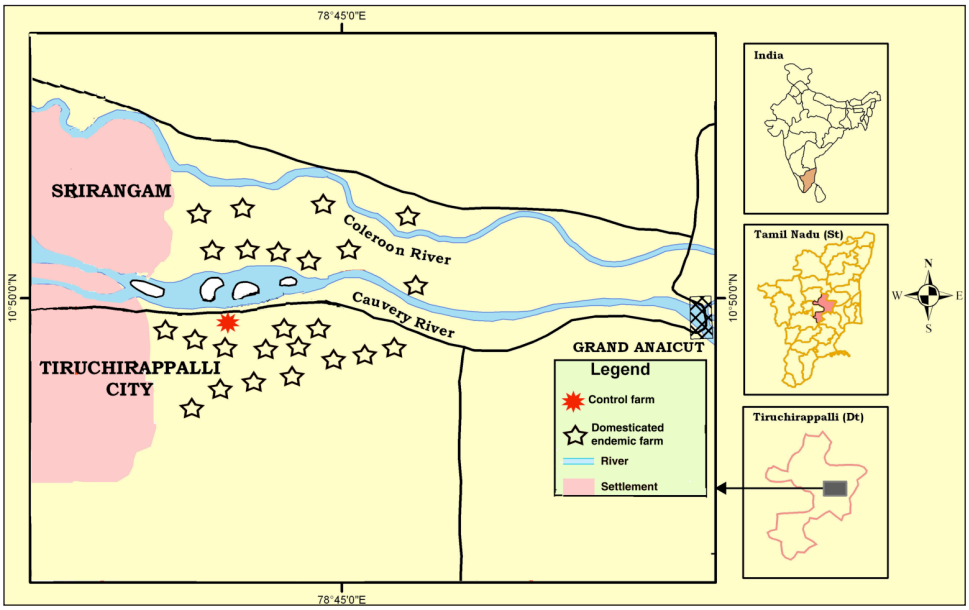

Fig 1–Map of study area in the Cauvery river valley in Tiruchirappalli, Tamilnadu, India.

workers and analyzed. In addition, sera and kidney samples from 321 field rats (*Rattus norvegicus*) caught in and around the farms were screened for leptospiral antibodies and *Leptospira* spp. A farm in the same area with a dairy protected by a wall and an ongoing rat control program was a source of sera from 73 cows, 89 rats, and 25 workers, and was included to determine the effects of reduced exposure to rats. Individual consents were obtained from farm workers and all procedures were approved by the Institutional Human and Animal Ethics Committee of Bharathidasan University.

Isolation of leptospires

One to two drops of urine from recently aborted cows were immediately inoculated into 5 ml of Ellinghausen, McCullough, Johnson, Harris (EMJH) (Difco, Franklin Lakes, NJ), semisolid medium and processed using standard procedures (Djordjevic *et al*, 1993). Inoculated media were transported within four hours to the laboratory where 0.5 ml aliquot was transferred to fresh EMJH.

Trapped field rats (*Rattus norvegicus*) were sacrificed by cervical dislocation and washed in 70% ethanol. The body cavity was then opened aseptically and pieces of kidney removed and inoculated in triplicate into EMJH as described earlier (Ratnam *et al*, 1987). EMJH contained 0.2% agarose (Sigma, St Louis, MO), 1% bovine serum albumin (Sigma), 2% rabbit serum, 0.1% sodium pyruvate (Sigma) and 100 g/ml 5-flurouracil. All cultures were incubated at 30°C in the dark, and examined weekly by dark field microscopy. Positive tubes were subcultured into fresh EMJH.

Microscopic agglutination test (MAT)

MAT was performed on all sera using 13 leptospiral reference serovars as follows: Australis (JezBratislava), Autumnalis (Bankinang), Ballum (Mus127), Bataviae (VanTienen), Canicola (HondUtrechIV), Grippotyphosa (MoskvaV),

Hebdomadis (Hebdomadis), Icterohaemorrhagiae (RGA), Javanica (Veldrat-Bataviae 46), Sejroe (Hardjoprajitno), Pomona (Pomona), Pyrogenes (Salinem), and Tarassovi (Perepilitsin) (Cole *et al*, 1973) All these serovars were from the Leptospira WHO Reference Center, Port Blair, India and maintained with periodic subculture in EMJH medium at the Department of Microbiology, Bharathidasan University, Tiruchirappalli, India. MAT was performed at doubling dilutions starting at 1:20. Seven-day-old cultures at a concentration of 1-2x10^8 organisms/ml were used as antigen (Cole *et al*, 1973). Positive cut-off values for bovine, human and rat sera were based on values in previous studies (Ratnam *et al*, 1983; Natarajaseenivasan and Ratnam, 1997).

Identification of serovar

A panel of 37 rabbit antisera representative of all pathogenic serogroups was used for MAT serogroup identification. Mouse monoclonal antibodies, F20C4, F98C8, F98C12, F98C19, F98C20 (WHO/FAO Collaborating Center for Reference and Research, KIT-Biomedical Research, Amsterdam, The Netherlands), were used to serogroup Javanica.

RAPD PCR profiling

Genomic DNA for PCR was extracted and purified (Boom *et al*, 1990). After two washes in 70% ethanol, DNA was dried and redissolved in Milli-Q water. A RAPD PCR profiling assay was performed as described by Gerritsen *et al* (1995) using primers B11 (5′-CCGGAAGAAGGGGC-GCCAT-3′) and B12 (5′-CGATTTAG AAGGACTTGCACAC-3′). For each RAPD assay, PCR was carried out at least three times in an Eppendorf Master Cycler (Germany). PCR products were analysed by electrophoresis in 1.5% horizontal agarose gels at 70V for 3 hours and stained for 20 minutes in ethidium bromide. Gel images were then recorded (Alpha Images, USA).

***secY* sequencing**

The translocase gene *secY* was targeted for PCR amplification because of its value for species identification using primers G1-5′-CTGAATCGCTGTATA-AAAGT-3′ and G2-GGAAAACAAAT-GGTCGGAAG (Gravekamp *et al*, 1993). PCR was carried out in a 50 l reaction mixture of 50 ng of purified DNA, 0.1 M of each primer, 250 M of each dNTPs (Genei, Bangalore, India), 3 mM $MgCl_2$, 0.5 U *Taq* DNA polymerase (NEB, Ipswich, MA), in 10 mM Tris-HCl (pH 9) and 50 mM KCl. Amplicons of interest were purified using Montage™ PCR centrifugal filters (Millipore, Billerica, MA) and sequenced by the single-extension method.

Statistical analysis

The significance of difference between seroprevalence on endemic farms and a farm practicing rat control was assessed by univariate analysis (chi-square).

RESULTS

Over 87% of dairy cows on the endemic farms were seropositive by MAT (cut-off titer of 1:80). Thirty-three point two percent of sera reacted with serovar Javanica, 22.1% with Autumnalis and 13.1% with Hardjo (Table 1).

Sera of 321 field rats were assayed for antibody to 13 serovars. Overall, 51.4% of these sera had titers ranging from 1:40 to 1:640 (cut-off = 1:40). Javanica was the most frequently reactive serovar with rat sera (50.3%) followed by Autumnalis (14.5%), Icterohaemorrhagiae (12.7%), and Pomona (9.7%) (Table 1). Seropositivity in 39 farm workers was 76.5% (cut-off titer = 1:80). MAT seropositivity ranged

Table 1
Serovars of *Leptospira* reactive in microscopic agglutination test (MAT) with sera of dairy cattle, field rats and farm workers.

Serovar	Dairy cattle		Field rats		Farm workers	
	% positive[a] (449/515)	Median titer	% positive[a] (165/321)	Median titer	% positive[a] (39/51)	Median titer
Australis	1.6	1:160	3.6	1:40	12.8	1:640
Autumnalis	22.1	1:320	14.5	1:80	43.6	1:640
Canicola	4.3	1:160	3.6	1:40	5.1	1:160
Grippotyphosa	0.8	1:80	1.8	1:40	2.6	1:80
Icterohaemorrhagiae	3.1	1:160	12.7	1:80	10.3	1:160
Javanica	33.2	1:640	50.3	1:80	17.9	1:640
Pomona	21.4	1:640	9.7	1:80	10.3	1:160
Hardjo	13.1	1:80	1.2	1:40	0.0	0
Pyrogenes	0.4	1:80	2.4	1:40	0.0	0

Positive cut-off titers were 1:80 for cow sera, 1:40 for rat sera and 1:80 for farm worker.
[a]Percents positive for each host may total more than 100 because of reactivities of some sera to more than one serovar.

from 1:80 to 1:5,120. Autumnalis was the predominant serovar reactive with these sera (43.6%) followed by Javanica (17.9%), Australis (12.8%), Icterohaemorrhagiae (10.3%), and Pomona (10.3%) (Table 1).

Cow sera from the enclosed dairy that practiced rodent control show a significantly ($p< 0.001$) reduced frequency of reactivity to serovar Autumnalis. Field rats trapped in this dairy exhibited a seropositivity of 34.8% to serovar Javanica and lower rates of positivity to Autumnalis and Icterohaemorrhagiae. A total of 13.1% of workers on this farm had serum MAT titers of 1:80 or 1:160. Autumnalis predominated among the serovars reactive with these sera.

Nineteen isolates of *Leptospira* spp were cultured from rat kidney and 4 from bovine urine. Thirteen rat isolates, propagated for identification by serology and DNA comparisons, showed morphology and motility characteristic of the genus *Leptospira* with optimum growth temperature between 18 and 30°C and reacted most strongly with antiserum to serogroup Javanica. Further screening against a panel of mAbs specific for serovar Javanica confirmed their identity as serovar Javanica (data not shown).

RAPD profiles of rat isolates were similar to each other and to *L. borgpetersenii* (Fig 2). These isolates clustered in bootstrap analysis were in the range 0.260 - 0.100 (Vedhagiri *et al*, 2009). The profiles of 6 other genome species, namely, *kirschneri, interrogans*, *noguchii*, *weilii*, *alexanderi* and *inadiae,* with the exception of *borgpetersenii,* were different from each other and from the rat isolates. Partial *secY* DNA sequence analysis of 285 bp amplicons from rat kidney tissue, cow urine and cow manure revealed 96% identity between the 3 amplicons and with *secY* of *L. borgpetersenii* serovar Javanica (Genbank accession numbers FJ434137, 38, 39).

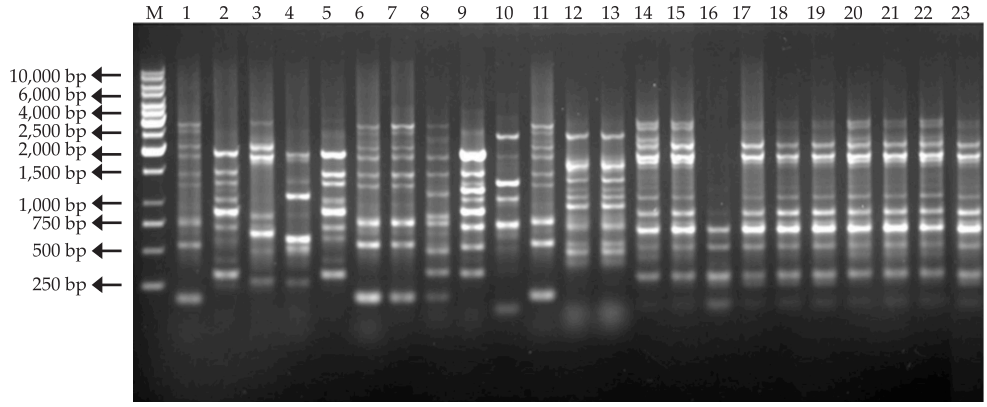

Fig 2–RAPD was carried out using primers B11 and B12 (Gerritsen *et al*, 1995) as described in Materials and Methods. Samples are (serotype, serovar, strain, genomospecies): lane 1: Bataviae, Bataviae, Swart, *L. interrogans*; lane 2: Canicola, Canicola, HondUtrech IV, *L. interrogans*; lane 3: Javanica, Javanica, Poi, *L. borgpetersenii*; lane 4: Ballum, Ballum, Mus 127, *L. borgpetersenii*; lane 5: Semaranga, Patoc, Patoc I, *L. biflexa*; lane 6: Cyanopteri, Cyanopteri, 3522 C, *L. kirschneri*; lane 7: Manhao, Manhao, L60, *L. alexanderi*; lane 8: Celledoni, Celledoni, Celledoni, *L. weilii*; lane 9: Hardjo, Sejroe, Hardjo prajitno, *L. interrogans*; lane 10: Louisiana, Louisiana, LSU 1945, *L. noguchii*; lane 11: Tarassovi, Tarassovi, Perepelician, *L. inadai*; lane 12: unidentified; lane 13: unidentified; lane 14: Javanica, Javanica, PMV12, *L. borgpetersenii*; lane 15: Javanica, Javanica, JDRM14, *L. borgpetersenii*; lane 16: Javanica, Javanica, PMV19, *L. borgpetersenii*; lane 17: Javanica, Javanica, PMV27, *L. borgpetersenii*; lane 18: Javanica, Javanica, JDRM22, *L. borgpetersenii*; lane 19: Javanica, Javanica, PMV31, *L. borgpetersenii*; lane 20: Javanica, Javanica, JDRM25, *L. borgpetersenii*; lane 21: Javanica, Javanica, JDRM33, *L. borgpetersenii*; lane 22: Javanica, Javanica, PMV47, *L. borgpetersenii*; lane 23: Javanica, Javanica, JDRM39, *L. borgpetersenii*. Strains PMV 12, 19, 27, 31, 47 were from rats trapped on endemic farms; strains JDRM 14, 22, 25, 33 and 39 were from rats on a farm with a program of rat control.

DISCUSSION

This study is the first to document the prevalence of leptospira antibody in unvaccinated dairy cattle experiencing a high incidence of abortion, infertility and stillbirths in the Cauvery river valley in Tamilnadu, Southeast India. The very high seroprevalence (87.2%) observed at 1:80 or above and evidence of urinary shedding post abortion suggest that leptospira infection is responsible for much of this loss. Surveys of seroprevalence in dairy herds in Europe and Canada with losses due to leptospira abortion/infertility have revealed much lower rates of seroprevalence (Atxaerandio *et al*, 2005; Peregrine *et al*, 2006). In the Basque region of Spain dairy cows have been shown to have a seroprevalence rate of 43.2 at a cut-off of 1:10. Reactive serovars were Pomona and Hardjo. In non-vaccinated dairy herds in Ontario, herds with sera reactive with serovars Hardjo, Icterohaemorrhagiae or Pomona totaled 45, 42 and 58%, respectively. Seroprevalence at 1:100 was 24.5% for serovar Hardjo, 19.5% for Icterohaemorrhagiae, and 24.2% for Pomona in these herds (Peregrine *et al*, 2006). Clearly, exposure to leptospira is much less intense

or of shorter duration in these herds than in cattle in the Cauvery valley.

Studies of bovine leptospirosis in different parts of the world indicate that serovars responsible for reproductive losses vary depending on which serovars are locally endemic. Serovars such as Hardjo (both genotypes), Pomona and Bratislava are well documented as causes of bovine abortion (Bolin, 2000). The predominance of antibody to serovar Javanica in dairy cattle of the Cauvery valley most probably reflected transmission from heavy populations of field rats (*Rattus norvegicus*), a species previously shown to be a carrier of serovar Javanica in Tamilnadu and implicated as a source of infection for sheep in that area (Natarajaseenivasan and Ratnam, 1999, 2000). Priya *et al* (2007) reported isolation of seorvars Autumnalis and Javanica from rats in Madurai and cited several reports documenting the widespread occurrence of serovar Javanica in rats and bandicoots in India (Ratnam *et al*, 1987).

In the present study it is unclear whether human seropositivity was derived mainly from direct exposure to rats or from contact with dairy cattle. Sera from dairy farm workers were 2.4 times more likely to show positivity to Autumnalis than to Javanica, whereas rat sera reacted predominantly with Javanica. Cow sera also showed a higher rate of positivity to Javanica than to Autumnalis. Possible explanations for differences in serovar specificity among the 3 groups of sera include greater infectivity or immunogenicity of Autumnalis for humans. This serovar produces severe disease in humans, whereas Javanica appears to be less virulent (Ratnam *et al*, 1983b). Although isolation of serovar Javanica was not attempted from aborted fetuses, this serovar is probably an important contributor to placental infection and fetal loss on dairy farms in the area. It is also probable that many human infections are derived from contacts with cattle or their feed during milking. Maceration of the skin of the hands and feet in environments where milking is conducted might also predispose farm workers to infection. The data from a single farm where rat control was practiced indicated that these measures reduced exposure to *Leptospira* spp. Finally, RAPD analysis confirmed that isolates of serovar Javanica from rats and cattle were probably clonal.

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