Original Article

Serovar prevalence of *Leptospira* in semirural India and the development of an IgM-based indirect ELISA

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Abstract

Introduction: Leptospirosis is a major public health problem in India. However, it has been underreported and under-diagnosed due to a lack of awareness of the disease, a functional surveillance system, and appropriate laboratory diagnostic facilities.

Methodology: This multicenter study aimed to understand the *Leptospira* serovars causing leptospirosis in seven secondary-level hospitals in six states in India. Since early and accurate diagnosis of leptospirosis is one of the challenges faced by clinicians in India due to the poor specificity and sensitivity of commercially available diagnostic systems, an in-house indirect enzyme-linked immunosorbent assay (ELISA) was developed. Genomic DNA from *L. interrogans* serovar Canicola was used for polymerase chain reaction amplification, cloning, and expression of the *lipL32* gene in *E. coli* to amplify, clone, and express the *lipL32* gene.

Results: Australis was the common serovar seen at all the study centers. Serovar Icterohaemorrhagiae was seen in samples from Tamil Nadu and Assam. In-house ELISA was standardized using the purified recombinant LipL32 polypeptide and was used to evaluate serum. Subsequently, acute serum samples from leptospirosis patients (n = 60) were screened. Compared to the gold standard, the microscopic agglutination test, sensitivity and specificity of the in-house ELISA was 95% and 90%, respectively.

Conclusions: Understanding *Leptospira* serovars circulating in leptospirosis-endemic areas will help to formulate better vaccines. LipL32-based ELISA may serve as a valuable tool for early diagnosis of leptospirosis.

Introduction

Leptospirosis, reportedly the commonest zoonoses in the world (especially in tropical countries), is an acute febrile illness caused by pathogenic spirochaetes of the genus *Leptospira*. Despite being a treatable disease, human leptospirosis is a significant public health problem and is severely neglected in its endemic hotspots of southern and western India [1]. Leptospires are classified into pathogenic, non-pathogenic, and intermediate species based on DNA-DNA hybridization. The old phenotypic classification system based on a cross-agglutination absorption test (CAAT) identified approximately 250 serovars among the *Leptospira* species and serogroups [2]. A serogroup defines a group of antigenically related serovars identified by the serological gold standard, the microscopic agglutination test (MAT). *Leptospira* taxonomy is complicated, and serovars in one serogroup may belong to different species [3]. Rodents, livestock, pets, and wildlife are reservoir hosts for leptospires [4]. Humans are incidental hosts and get infected by exposure to an environment contaminated with the urine of an animal reservoir. Animals are reservoirs for certain serovars and incidental hosts for others. Presence of serovars varies depending on local animal species and adaptability of serovars to new hosts [5]. Rats are maintenance hosts of serovar Icterohaemorrhagiae, cattle of Hardjo and Pomona, pigs of Pomona or Tarrossovi, and dogs of Canicola [6]. Emergence of serovars is attributed to introduction of new hosts in an area, adaptation to these hosts, and local ecological changes [7]. As clinical diagnosis is difficult, early laboratory diagnosis of leptospirosis is important [8]. Isolation of leptospires is time consuming and has

low sensitivity. MAT, the reference serological test, requires technical expertise, can be done only in reference laboratories that maintain live *Leptospira* strains, and is best interpreted with both acute and convalescent sera [9]. Most clinical microbiology laboratories employ commercial IgM ELISA for routine testing. These assays use whole cell lysates broadly reactive with pathogenic and nonpathogenic leptospires. LipL32, an outer membrane immunodominant protein expressed only in pathogenic leptospires during active infection, is emerging as an excellent candidate for early diagnosis of leptospirosis [10].

A functional surveillance system based on identification of serovars from humans and animals is essential for leptospirosis control and vaccine development. Identification of the infecting serovar can be done by isolation or MAT. MAT uses a panel of leptospiral strains as antigens to detect agglutinating antibodies [11]. Vaccines currently used against leptospirosis are prepared using two or more prevalent serovars and confer serovar-specific immunity [12]. Routine surveillance of locally circulating serovars is important for appropriate formulation of the vaccine.

The diversity and distribution of leptospiral serovars in India remains largely unexplored. A multicenter project was carried out to determine etiological agents of acute undifferentiated fever (AUF) in seven secondary level hospitals in six states in India. The study, a sub-study of the project, had two objectives: to identify circulating *Leptospira* serovars at the study centers, and to develop an in-house ELISA as an alternative to the commercial ELISA commonly used at the study centers. All samples were screened for anti-leptospira IgM by a commercial ELISA. Samples positive by screening ELISA were tested by MAT to identify serovars. LipL32 was expressed, purified, and evaluated for the diagnosis of leptospirosis from single acute-phase serum samples.

Methodology
Study centers and participants
The study was conducted from April 2011 to November 2012. All patients > 5 years of age admitted with AUF for 2–14 days were recruited from 7 secondary hospitals located at Ambur (Bethesda Hospital) and Oddanchatram (Christian Fellowship Hospital) in Tamil Nadu, Anantapur (Rural Development Trust) in Andhra Pradesh, Ratnagiri (BKL Walawalkar Hospital) in Maharashtra, Mungeli (Christian Hospital) in Chattisgarh, Raxaul (Duncan

Hospital) in Bihar, and Tezpur (Baptist Christian Hospital) in Assam.

The Benjamin M. Pulimood Laboratory for Infection, Inflammation and Immunity (BMPLIII), Department of Medicine-1 and Infectious Diseases, Christian Medical College, Vellore was the coordinating center.

Samples for MAT
MAT was performed on samples positive (n = 185), discrepant (n = 7), equivocal (n = 3), and negative (n = 51) by screening ELISA.

Samples for evaluation of in-house indirect ELISA
Healthy donor samples (n = 27) were used for the standardization of the indirect in-house ELISA. Serum samples (Total, n = 60; ELISA & MAT positive, { n = 40}) from patients with confirmed diagnosis of leptospirosis were used for evaluating the in-house ELISA. Twenty samples negative by both screening ELISA and MAT were used as controls.

Plasmids
A genomic DNA sample of *L. interrogans* serovar Canicola (provided by *Leptospira* Research Centre, Tamil Nadu Veterinary and Animal Sciences University, Chennai) was used for the study. Expression plasmid pRSETA (Invitrogen, Carlsbad, USA) and *E. coli* strain DH5α were used for cloning. BL21(DE3) (Novagen, Madison, USA) was the host system for protein expression. The expressed LipL32 was purified through the IMAC Hypercel (PALL Life Sciences, New York, USA), and the purified antigen was confirmed with antibodies raised in a mouse (Sigma-Aldrich, St. Louis, USA).

Screening
First-line screening for leptospirosis was performed on single acute-phase serum samples using a commercially available *Leptospira* IgM ELISA (Panbio Pty., Ltd., Queensland, Australia), an indirect ELISA detecting antibodies to pre-coated *Leptospira* antigen. The testing was done per the manufacturer's instructions and was performed at the study hospitals. Samples untested at the study sites were tested at the coordinating laboratory at Christian Medical College, Vellore.

MAT
Testing was performed on ELISA positive (n = 185), discrepant (n = 7), equivocal (n = 3), and negative (n = 51) samples per World Health Organization

(WHO) standards at the Zoonoses Research Laboratory, Centre for Animal Health Studies, Madhavaram, Chennai. *Leptospira* serovars used included Australis (As), Autumnalis (At), Pyrogenes (Py), Ballum (B), Grippotyphosa (G), Javanica (Ja), Hardjo (H), Tarrosovi (Ta), Pomona (Po), Hebdomadis (He), Icterohaemorrhagiae (Ic), and Canicola (Ca). The endpoint was defined as the highest dilution of serum which agglutinated 50% of the leptospires, and an agglutination titer of 100 was considered reactive.

Polymerase chain reaction (PCR)

The genomic DNA of *Leptospira interrogans* serovar Canicola was used to amplify the *lipL32* gene by PCR using published primers [13]. The reaction was carried out in a 25 µL reaction mixture containing 10X PCR buffer with $MgCl_2$, 20 pmol of each primer, 0.2 mM dNTPs, 5 units of Vent polymerase, and 20 ng of template DNA. The following cycling conditions were used: initial denaturation at 95°C for 2 minutes followed by 30 cycles each of denaturation 95°C for 30 seconds, primer annealing at 56°C for 45 seconds, primer extension at 72°C for 60 seconds, and final extension at 72°C for 10 minutes. The amplified DNA products (790 base pairs) were visualized using a UV transilluminator (Mighty Bright, Hoeffer Scientific Instruments, San Francisco, USA). The PCR product was eluted and used for gene sequencing with forward and reverse primers.

Cloning and expression

The amplicon of the *lipL32* gene was cloned into *Xho* 1 and *Hind* III (New England Biolabs, Boston, USA) sites of the pRSETA vector containing His_6 tag. The cloned plasmid was transformed into the expression host *E. coli* BL21DE3 (Novagen Madison, USA) using the $CaCl_2$ method. A single colony was inoculated into Luria-Bertani (LB) medium (HiMedia Lab, Mumbai, India) containing 100 mg/mL ampicillin and grown at 37°C until optical density (OD) at 600 nm reached 0.6. The bacterial culture was induced for the expression of recombinant protein by the addition of 2mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma-Aldrich, St. Louis, USA) at 37°C. From the samples collected at different time points of induction, the cells were harvested by centrifugation at 14,000 rpm for 10 minutes and resuspended in lysis buffer. The lysed cell suspension was centrifuged at 14,000 rpm for 15 minutes at 4°C to separate soluble fraction of protein from inclusion bodies. Protein concentration was estimated by Bradford's method, with bovine serum albumin as a standard.

Protein expression and SDS analysis

Sodium deodecyl sulphate (SDS)-poly acrylamide gel electrophoresis (PAGE) was carried out to analyze the expressed protein. Equal amounts of the soluble and insoluble fractions of protein samples were mixed with 2 × SDS sample loading buffer and boiled for 10 minutes. The samples were run on 12% SDS-PAGE gels at 90 V using Bio-Rad mini protein system (Biorad, Philadelphia, USA) The resolved protein samples were visualized by staining with Coomassie brilliant blue (Sigma-Aldrich, St. Louis, USA)

Protein purification and western blotting

The expressed LipL32 protein was then purified by immobilized metal ion affinity chromatography (IMAC) using IMAC Hypercel (Pall Life Sciences, New York USA), which was charged with nickel ion and was confirmed through 12% SDS PAGE gel followed by western blot using monoclonal anti-poly His antibody raised in mouse. The proteins resolved on a 12% SDS-PAGE gel were transferred to nitrocellulose membrane for 90 minutes at 90 V in transfer buffer using a Bio-Rad mini transblot apparatus (Bio-Rad, Philadelphia, USA). After blocking with 5% skimmed milk powder, the membrane was washed three times with phosphate-buffered saline with Tween-20 (PBST), then incubated with monoclonal anti-poly histidine antibody for 1 hour and then with anti IgG (secondary antibody) conjugated with alkaline phosphatase. The positive reactivity was visualized by using 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetra-zolium (BCIP-NBT, Sigma-Aldrich, St. Louis, USA) as a substrate.

Standardization of in-house indirect ELISA

Standardization was done using healthy donor samples (n = 27) with appropriate controls. Endpoint titration was performed to determine the working dilutions of primary antibody and conjugate. Cut-off values were calculated using mean ± 2 standard deviations (SD). Serum samples (n = 60) were used to evaluate rLipL32-based indirect in-house ELISA. rLipL32 antigen was coated on polystyrene microtiter plates (Nunc Polysorp, Nalge Nunc International, Rochester, USA) using phosphate buffer saline (pH 9.6) and incubated overnight at 4°C. Post-incubation, the plates were washed thrice with PBST. Blocking buffer was then added to block the uncoated sites and incubated at 37°C. Post incubation and wash, patient sera (1:100) was added. Horseradish peroxidase-conjugated anti-human IgM (Sigma-Aldrich, St. Louis, USA) (1: 25,000) was the secondary antibody. OD was

measured at 450 nm after addition of substrate. Sensitivity and specificity were calculated against the reference standard, MAT.
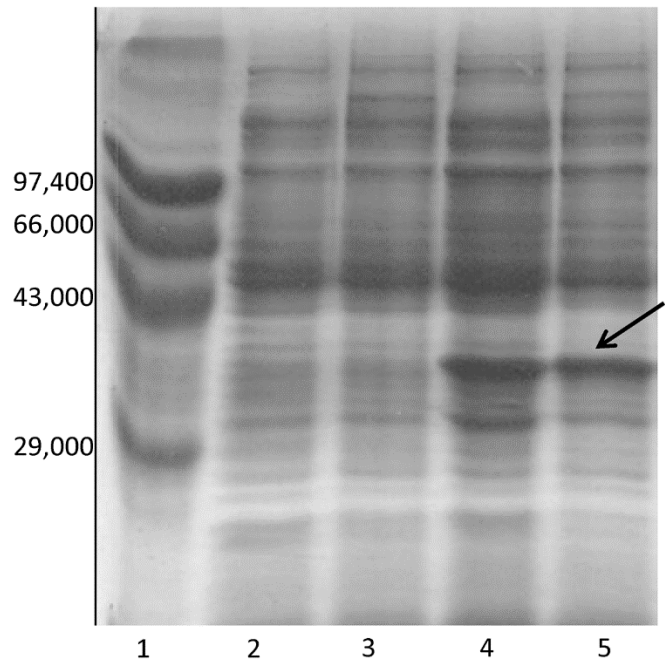
Results

As part of the AUF study, samples from participating centers were screened for anti-*Leptospira* IgM (Panbio IgM) ELISA. Screening ELISA positive (n = 185), discrepant (n = 7), equivocal (n = 3), and negative (n = 51) isolates were tested by the MAT (n = 246) and included samples from Ambur (n = 47), Oddanchatram (n = 16), Ratnagiri (n = 54), Tezpur (n = 80), Anantpur (n = 22), Raxual (n = 17), and Mungeli (n = 10) (Table 1).

Serovar epidemiology

Of the samples reactive by MAT (n = 162), 135 (83%) were reactive to a single and 27 (17%) to more than one serovar. Among MAT positives, 33% (n = 54) were reactive to serovar Australis, 14.8% (n = 24) to Pyrogenes, 8.6% (n = 14) to Autumnalis, 8% (n = 13) to Javanica, 7% (n = 12) to Tarossovi, and 6% (n = 10) to Pomona. Serovars Hebdomadis, Icterohaemorrhagiae, and Canicola were represented in 2.5% (n = 4), 2% (n = 3), and 0.6% (n = 1) samples, respectively (Table 2). Serovar Hardjo was not detected in any of the samples. Serovars Ballum and

Figure 1. SDS- poly acrylamide gel electrophoresis (PAGE) gel stained with Coomassie blue.

Lane 1: protein marker; lane 2: pRSETA uninduced; lane 3: pRSETA induced; lane 4: uninduced bacterial lysate; lane 5: IPTG-induced bacterial lysate.

Table 1. ELISA and MAT results from the study sites.

	MAT +	MAT-	Total
ELISA positive (Total)	121	64	
Ambur	15	1	
Oddanchatram	9	3	
Anantpur	14	4	
Ratnagiri	27	19	185
Raxaul	5	7	
Mungeli	2	3	
Tezpur	49	27	
ELISA negative (Total)	31	20	
Ambur	20	4	
Oddanchatram	2	2	
Anantpur	1	3	
Ratnagiri	4	3	51
Raxaul	2	2	
Mungeli	1	3	
Tezpur	1	3	
ELISA equivocal (Total)	3	0	
Ratnagiri	1	0	
Raxaul	1	0	3
Mungeli	1	0	
ELISA discrepant (Total)	7	0	
Ambur	7		7
TOTAL	**162**	**84**	**246**

ELISA: enzyme-linked immunosorbent assay; MAT: microscopic agglutination test.

Table 2. Serovar distribution across the study sites.

Serovars	Ambur	Oddanchatram	Anantpur	Ratnagiri	Raxual	Mungeli	Tezpur	N (%)
				Centers				
Australis	20	5	6	9	3	1	10	54 (33)
Autumnalis	5	1	1	1	0	0	6	14 (8.6)
Pyrogenes	6	1	3	8	0	0	6	24 (14.8)
Javanica	1	0	4	3	2	1	2	13 (8)
Tarrosovi	2	2	1	4	0	0	3	12 (7)
Pomona	1	0	0	6	1	0	2	10 (6)
Hebdomadis	0	0	0	0	0	1	3	4 (2.5)
Icterohaemorrhagiae	1	2	0	0	0	0	0	3 (1.9)
Canicola	1	0	0	0	0	0	0	1 (0.6)
Mixed	5	0	0	1	2	1	18	27 (17)
Total	**42**	**11**	**15**	**32**	**8**	**4**	**50**	**162**

Grippotyphosa were detected only in mixed infections from Mungeli and Tezpur, respectively. Serovar Icterohaemorrhagiae was identified only from Tamil Nadu and Tezpur. Infection with multiple serovars was seen predominantly (67%; n = 18) in Tezpur samples.

PCR amplification and cloning

The extracted genomic DNA from *Leptospira interrogans* serovar Canicola showed as a 790 bp gene fragment on 1% agarose gel. Sequence analysis revealed a 99% homology with pathogenic serovars of *Leptopsira*. Gel electrophoresis of the DNA amplicon of recombinant pRSETA carrying LipL32 revealed a 790 bp band and confirmed the presence of the plasmid containing the LipL32 insert.

Protein expression and SDS-PAGE analysis

LipL32 protein expression was induced in *E. coli* BL21DE3 in LB medium containing ampicillin. Protein expression was done with 2mM IPTG at 37°C. Subsequent analysis of non-purified fraction by SDS-PAGE revealed a band of ~32 kDa (Figure 1).

Purification of LipL32

The expressed protein was purified using IMAC-Hypercel –Ni^{2+}. The chromatogram of IMAC showed different peaks at pH 7, 6, 5, and 4 with pH gradient elution. SDS page analysis was performed for the fraction eluted at pH 7, 6, 5, and 4. The peak eluted at pH 4 shows the presence of a highly purified protein band at 32 kDa (Figure 2) and subsequent immunoblot analysis with monoclonal anti-poly histidine antibody raised in a mouse (Figure 3).

Figure 2. Purification of expressed protein, LipL32. **a)** IMAC - Hypercel-Nickel Chromatogram which represents the protein peaks at different pH. **b)** SDS- PAGE gel analysis of purified recombinant LipL32.
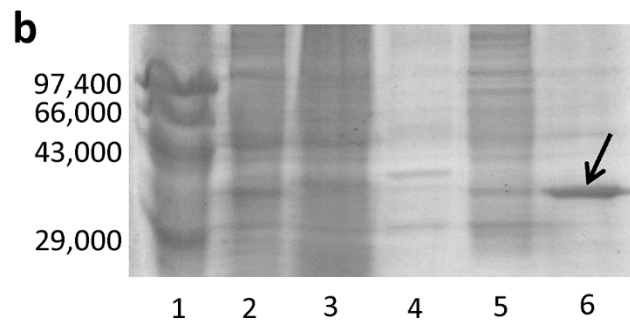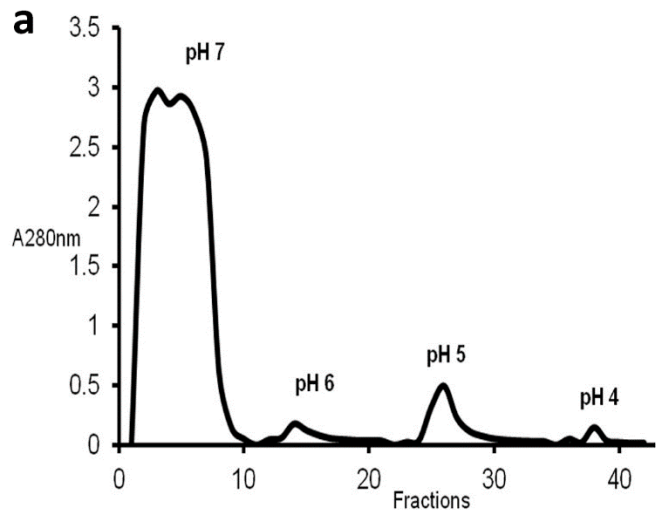
Lane 1: protein marker; lane 2: crude fractions (pH7); lane 3: non-retained fractions (pH 7); lane 4: protein eluted at pH 6; lane 5: protein eluted at pH 5; lane 6: protein eluted at pH 4.

Figure 3. Western blot analysis of LipL32.
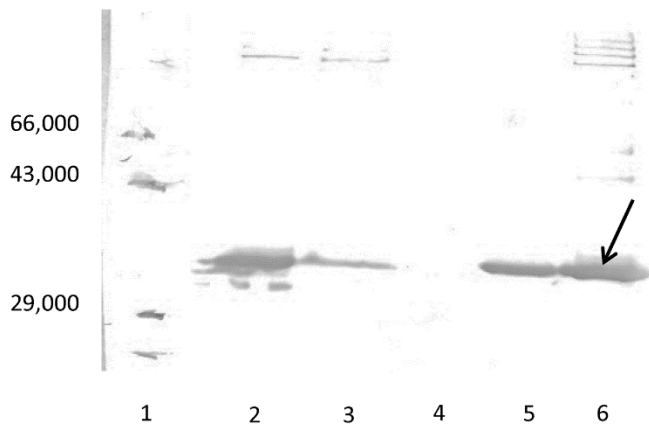
Lane 1: protein marker; lane 2: crude fractions (pH7); lane 3: non-retained fractions (pH 7); lane 4: protein eluted at pH 6; lane 5: protein eluted at pH 5; lane 6: protein eluted at pH 4.

Recombinant LipL32-based ELISA

The in-house ELISA was standardized with different concentrations of antigen and primary and secondary antibody. Based on the healthy donor sera, the cut-off was 0.4 OD units. The sensitivity and specificity of rLipL32 compared to the reference test MAT was 95% and 90%, respectively (Table 3).

Discussion

Leptospirosis, though endemic in many parts of India, is underreported, and the circulating serovars remain unknown. The absence of surveillance systems and appropriate laboratory support are key factors in persistence of the disease. Leptospirosis hotspots are in Tamil Nadu (Chennai), Maharashtra (Mumbai), Kerala, Gujarat, and Andamans. Serovars reportedly circulating include Icterohaemorrhagiae, Australis, Autumnalis, and Javanica in Andhra Pradesh and Icterohaemorrhagiae, Bataviae, Tarrossovi, Canicola, Australis, and Pomona in Maharashtra [14]. Serovars Australis, Pyrogenes, Canicola, and Hebdomadis have been reported from Tamil Nadu. Serovars Copenhageni, Autumnalis, Pyrogenes, Grippotyphosa, Canicola, Australis, Javanica, Sejroe, Louisiana, and Pomona have been reported from other states [15]. In this study, we identified, for the first time, *Leptospira* serovars circulating in Assam (northeast India), Chattisgarh (central India), and Raxaul (northern India). Overall, serovar Australis was detected from all sites. In areas of high endemicity, as in Tezpur, co-infection with multiple serovars was common, as reported in an earlier study [16].

Definite diagnostic tests for leptospirosis are culture and MAT. However, culture is insensitive and requires

Table 3. Sensitivity and specificity of rLipL32 ELISA compared to the gold-standard MAT.

MAT	LipL32 ELISA	
	Positive	Negative
Positive	38	2
Negative	2	18

ELISA: enzyme-linked immunosorbent assay; MAT: microscopic agglutination test.

several weeks of incubation, which limits its use in diagnostic laboratories. MAT is a serogroup/serovar-specific test and is carried out with suspensions of living cultures or cultures inactivated with neutralized formaldehyde [17]. It provides important epidemiological information on probable circulating serovars. MAT gives a presumptive idea of which serogroups may be present in a population, and conclusions about circulating serogroups/serovars can only be made with isolation. Cross-reactivity between serogroups and paradoxical reactions are common. A high degree of cross-reaction occurs between different serogroups, especially in acute-phase sera. Serogroup specificity is relatively higher in the convalescent phase. Unavailability of convalescent phase sera was a limitation of the study [18].

Most tertiary care centers in India use the commercially available ELISA (PanBio *Leptospira* IgM ELISA, PanBio, Queensland, Australia) [19] for laboratory diagnosis of leptospirosis. It is a genus-specific test. Serovar identification by isolation and/or MAT is done only in reference centers.

The study sites in Tamil Nadu, Ambur, and Oddanchatram predominantly showed circulation of serovar Australis. Serovars Icterohaemorrhagiae and Canicola were detected only in samples from these sites. During 2004–2005, Australis was the predominant serovar in Chennai, followed by Pyrogenes, Grippotyphosa, Autumnalis, and Hebdomadis. Serovar Canicola reportedly replaced Grippotyphosa during 2005–2006. Serovar Tarassovi, which earlier had been reported in wild animals, was detected from both sites [15].

Ratnagiri in Maharashtra is endemic for leptospirosis. Serovars Australis, Pyrogenes, Pomona, Javanica, and Tarassovi were detected in our study. Serovars seen from Anantpur in Andhra Pradesh were Australis, Javanica, and Pyrogenes. Serovars Australis and Javanica were detected from Bihar and Chattisgarh. Additionally, Pomona and Hebdomadis were also detected from the two sites, respectively. To the best of our knowledge, this is the first report of circulating serovars from Bihar, Chattisgarh, and Assam. Serovars Australis, Pyrogenes, Autumnalis, Tarassovi,

Hebdomadis, Javanica, and Pomona are circulating in Tezpur. Multiple serovars were detected in many (22%) Tezpur cases. Characterization of serovars helps define local antigens to be used for MAT. Distribution of serovars has implications for vaccine design and efficacy [20]. Epidemiological evidence will lead to inclusion of prevalent serovars in animal vaccines. Knowledge of prevalent serovars and their animal host is important in understanding the epidemiology of the disease [21].

The role of LipL32 in diagnosis of leptospirosis in human and animals has been reported [22]. LipL32 is the most widely studied protein in *Leptospira* research due to its vital role in pathogenesis [23]. About 95% of patients infected with leptospires produce antibodies against LipL32. It is considered a major candidate for vaccine development. IgM antibodies are directed against the C terminal domain of LipL32 during acute and convalescent infection. Developing an ELISA that detects the IgM-specific antibodies against *Leptospira* would help in early identification of infection [23]. In our study, recombinant LipL32 from pathogenic *L. interrogans* was cloned and expressed. Expressed recombinant protein was purified and was used to standardize an in-house ELISA. The *lipL32* gene was His$_6$ tagged, as it reportedly helps in efficient purification by IMAC, facilitating high protein yield, proteolysis prevention, and increased solubility. Sensitivity and specificity of the ELISA-based assay developed using rLipL32 was 95% and 90%, respectively, when compared to the gold-standard MAT. Studies from India have reported sensitivity and specificity of rLipL32-based ELISA as 96.4% and 90.4%, respectively. The lower specificity (90%) of rLipL32 ELISA could be due to the limited number of serovars used for the MAT [24]. Our study demonstrates that the rLipL32-based ELISA developed has good diagnostic accuracy. However, these results are preliminary and more samples must be tested before using this in-house ELISA for routine diagnosis. Overall, this study was multi-pronged and helped determine the serovars circulating in various parts of India. The agreement of commonly used commercial serological assays with reference serological standard was analyzed, and an alternative to commercial assays was explored. The study provides valuable insight into an underreported, often-encountered public health problem in India.

Conclusions

Leptospirosis remains a neglected public health problem in India on account of the poor diagnostic systems used and the absence of important epidemiological data. Vaccines currently in use confer serovar-specific immunity. Presumptive information on local circulating serovars should guide the formulation of effective vaccines. The study presents preliminary serovar data from certain neglected leptospirosis hotspots in India. In resource-starved settings, early detection and treatment of leptospirosis should remain priority areas of research. The rLipL32-based approach has shown promising results in this study and should be explored further.

References
1. Kamath SA, Joshi SR (2003) Re-emerging of infections in urban India – focus Leptospirosis. J Assoc Physicians India 51: 247-248.
2. Brenner DJ, Kaufmann AF, Sulzer KR, Steigerwalt AG, Rogers FC, Weyant RS (1999) Further determination of DNA relatedness between serogroups and serovars in the family Leptospiraceae with a proposal for *Leptospira alexanderi* sp. nov. and four new *Leptospira* genomospecies. Int J Syst Bacteriol 49: 839-858.
3. Everard COR, Edwards CN, Everard JD, Carrington DG (1995) A twelve-year study of leptospirosis on Barbados. Eur J Epidemiol 11: 311-320.
4. Martins G, Lilenbaum W (2013) The panorama of animal leptospirosis in Rio de Janeiro, Brazil, regarding the seroepidemiology of the infection in tropical regions. BMC Vet Res 9: 237.
5. Martins G, Penna B, Lilenbaum W (2012) The dog in the transmission of human leptospirosis under tropical conditions: victim or villain? Epidemiol Infect 140: 207-208.
6. Goldstein RE (2010) Canine leptospirosis. Vet Clin Small Anim 40: 1091-1101.
7. Faine S (1999) Leptospira and leptospirosis, 2nd edition. Melbourne: MediSci. 272 p.
8. Seijo A, Coto H, San Juan J, Videla J, Deodato B, Cernigoi B, Messina OG, Collia O, de Bassadoni D, Schtirbu R, Olenchuk A, de Mazzonelli GD, Parma A (2002) Lethal leptospiral pulmonary hemorrhage: an emerging disease in Buenos Aires, Argentina. Emerg Infect Dis 9: 1004-1005.

9. Myers DM (1976) Effect of culture medium on the agglutination of leptospires by microscopic agglutination test. Rev Agent Microbiol 8: 14-20.

10. Haake DA, Chao G, Zuerner RL, Barnett JK, Barnett D, Mazel M, Matsunaga J, Levett PN, Bolin CA (2000) The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. Infect Immun 68: 2276-2285.

11. Winslow WE, Merry DJ, Pirc ML, Devine PL (1997) Evaluation of a commercial enzyme-linked immunosorbent assay for detection of immunoglobulin M antibody in diagnosis of human leptospiral infection. J Clin Microbiol 35: 1938-1942.

12. Palaniappan RU, Ramanujam S, Chang YF (2007) Leptospirosis: pathogenesis, immunity, and diagnosis. Curr Opin Infect Dis 20: 284-292.

13. Hauk P, Carvalho E, Ho PL (2011) Expression and purification of the non-tagged LipL32 of pathogenic Leptospira. Braz J Med Biol Res 44: 297-302.

14. Velineni S, Asuthkar S, Umabala P, Lakshmi V, Sritharan M (2007) Serological evaluation of leptospirosis in Hyderabad, Andhra Pradesh: A retrospective hospital-based study. Indian J Med Microbiol 25: 24-27.

15. Koteeswaran A (2006) Seroprevalence of Leptospirosis in man and animals in Tamilnadu. Indian J Med Microbiol 24: 329-331.

16. Kalita JB, Rahman H (2008) Leptospirosis among patients with pyrexia of unknown origin in a hospital in Guwahati, Assam. Indian J Public Health 52: 107-109.

17. Wolff JW (1954) The laboratory diagnosis of leptospirosis. Springfield: University of Michigan. 99 p.

18. Musso D, La Scola B (2013) Laboratory diagnosis of leptospirosis: a challenge. J Microbiol Immunol Infect 46: 245-252.

19. Bajani MD, Ashford DA, Bragg SL, Woods CW, Aye T, Spiegel RA, Plikaytis BD, Perkins BA, Phelan M, Levett PN, Weyant RS (2003) Evaluation of four commercially available rapid serologic tests for diagnosis of Leptospirosis. J Clin Microbiol 41: 803-809.

20. Dong H, Hu Y, Xue F, Sun D, Ojcius DM, Mao Y, Yan J (2008) Characterization of the ompL1 gene of pathogenic Leptospira species in China and cross-immunogenicity of the OmpL1 protein. BMC Microbiol 8: 223.

21. Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, Levett PN, Gilman RH, Willig MR, Gotuzzo E, Vinetz JM (2003) Peru-United States Leptospirosis Consortium. Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis 3: 757-771.

22. Flannery B, Costa D, Carvalho FP, Guerreiro H, Matsunaga J, Da Silva ED, Ferreira AG, Riley LW, Reis MG, Haake DA, Ko AI (2001) Evaluation of Recombinant Leptospira antigen-based enzyme-linked immunosorbent assays for the serodiagnosis of leptospirosis. J Clin Microbiol 39: 3303-3310.

23. Hauk P, Guzzo CR, Roman Ramos H, Ho PL, Farah CS (2009) Structure and calcium-binding activity of LipL32, the major surface antigen of pathogenic Leptospira sp. J Mol Biol 390: 722-736.

24. Vedhagiri K, Velineni S, Timoney JF, Shanmughapriya S, Vijayachari P, Narayanan R, Natarajaseenivasan K (2013) Detection of LipL32-specific IgM by ELISA in sera of patients with a clinical diagnosis of leptospirosis. Pathog Glob Health: 130-135.

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Conflict of interests: No conflict of interests is declared.