



Contents lists available at ScienceDirect

Journal of King Saud University – Science

journal homepage: [www.sciencedirect.com](http://www.sciencedirect.com)

Original article

# Isolation and identification of *Leptospira* species from bovines by *rpoB* and *LipL41* genes based phylogenetic analysis

Shivaraj Murag<sup>a</sup>, Doddamane Rathnamma<sup>b</sup>, Vinayagamurthy Balamurugan<sup>c</sup>, Sharanagouda Patil<sup>c</sup>, Mudalagiri Dasappa Venkatesha<sup>a</sup>, Raghavendra G. Amachawadi<sup>d</sup>, Asad Syed<sup>e</sup>, Najat Marraiki<sup>e</sup>, Sandeep Kumar Singh<sup>f,g</sup>, Shiva Prasad Kollur<sup>h,\*</sup>, Chandan Shivamallu<sup>i,\*</sup><sup>a</sup> Institute of Animal Health and Veterinary Biologicals, KVAFSU, Hebbal, Bengaluru 560024, India<sup>b</sup> Department of Microbiology, Veterinary College, KVAFSU, Hebbal, Bengaluru 560024, India<sup>c</sup> ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), Yelahanka, Bengaluru 560064, India<sup>d</sup> Department of Clinical Sciences, College of Veterinary Medicine, Kansas State University, Manhattan, KS, USA<sup>e</sup> Department of Botany and Microbiology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia<sup>f</sup> Centre of Biomedical Research (CBMR), SGPGI Campus, Lucknow, U.P, India<sup>g</sup> Indian Scientific Education and Technology (ISET) Foundation, Lucknow, India<sup>h</sup> Department of Sciences, Amrita School of Arts and Sciences, Amrita Vishwa Vidyapeetham, Mysuru Campus, Mysuru, Karnataka 570 026, India<sup>i</sup> Department of Biotechnology and Bioinformatics, School of Life Sciences, JSS Academy of Higher Education & Research, Mysuru 570015, India

## ARTICLE INFO

### Article history:

Received 6 October 2020

Revised 16 November 2020

Accepted 29 November 2020

Available online 5 December 2020

### Keywords:

*Leptospira*

MAT

*LipL41**rpoB*

Phylogenetic analysis

## ABSTRACT

In this study, in the beginning 582 serum samples were subjected to Microscopic Agglutination Test (MAT) with eight different serovars prevalent in the region to know the seroprevalence of *Leptospira* in bovines in Karnataka, India. Based on the findings of the MAT, different samples like blood, urine, aborted materials and uterine discharge collected from the MAT positive animals were used for isolation and genomic detection by conventional PCR targeting two *lipL32* and *seqY* genes using specific primers. Out of the 163 MAT positive samples screened 12 samples (including three isolates) were found positive in PCR. Subsequently, to identify the different species prevalent in the geographical region the PCR positive samples were subjected to *rpoB* and *LipL41* gene amplification. and nucleotide sequence analysis of *rpoB*, it was found that all the samples were belonging to *L. interrogans* species with overlapping/superimposing *L. interrogans* and *L. borgpetersenii* species. Further, the *LipL41* gene sequence phylogenetic analysis differentiated these two species clearly. Therefore, it can be concluded that *LipL41* gene based phylogenetic analysis besides *rpoB* gene can be effectively utilized to identify different *Leptospira* species in a geographical niche including the identification of intermediate species. This is first of its kind in India using *LipL41* gene based phylogenetic analysis for *Leptospira* species identification in limited number of samples from bovines, hence the same can be explored on a larger geographical area with more number of samples and even to identify the prevalence or presence of intermediate species in different geographical locations.

© 2020 The Author(s). Published by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

\* Corresponding authors.

E-mail addresses: [agravh@vet.k-state.edu](mailto:agravh@vet.k-state.edu) (R.G. Amachawadi), [assyed@ksu.edu.sa](mailto:assyed@ksu.edu.sa) (A. Syed), [najat@ksu.edu.sa](mailto:najat@ksu.edu.sa) (N. Marraiki), [sandeeps.bhu@gmail.com](mailto:sandeeps.bhu@gmail.com) (S. Kumar Singh), [shivachemist@gmail.com](mailto:shivachemist@gmail.com) (S. Prasad Kollur), [chandans@jssuni.edu.in](mailto:chandans@jssuni.edu.in) (C. Shivamallu).

Isolation and Identification of *Leptospira* species from bovines by *rpoB* and *LipL41* genes based phylogenetic analysis by Shivaraj Murag et al. 2020.



Production and hosting by Elsevier

## 1. Introduction

Leptospirosis, a bacterial disease of zoonotic importance, prevalent globally and affects most of the mammalian species including both domestic and wild animals. Many leptospira serovars are isolated even from aquatic species. The disease spreads through multiple routes both directly or indirectly, however contaminated soil or water plays a crucial role in transmission of the disease (Hartskeerl and Terpstra, 1996). According to World Health Organization (WHO, 1999), in India huge and diversified animal population with their close contact with farming community is playing an important role in the spread of the disease. Apart from this, poor sanitary conditions, poor animal management and close

<https://doi.org/10.1016/j.jksus.2020.101272>

1018-3647/© 2020 The Author(s). Published by Elsevier B.V. on behalf of King Saud University.

This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

association between man and animals provide a favourable environment for the spread of the disease. Leptospirosis occurs round the year with a high incidence during rainy season due to the survival of the *Leptospira* for a quite long time in the logged water conditions and slightly alkaline environment (Levett, 2001).

Among domestic animals, bovine leptospirosis has been associated with serious financial loss due to infertility, abortions, high culling rates, mastitis or decrease in milk yield. Most of the times the cattle remains as symptomless carrier of leptospires for long periods and pose a great threat to other healthy animals and working personnel. The abortions with leptospirosis are due to interference with the implantation of embryo or with other pregnancy events (Dhaliwal and Murray, 1996; Guitian et al., 1999).

Routinely, in the laboratory, the leptospirosis is diagnosed based on either antigen or antibody detection. However, detection of *Leptospira* serovar by serology like Microscopic Agglutination Test (MAT) and ELISA needs biosafety and biosecurity laboratories. Therefore, in identification of leptospirosis conventional Polymerase Chain Reaction (PCR) for antigen detection plays a significant role in detection of types of serovar/serogroup involved in the given outbreak or prevalent in that geographical area. In this context, various genes from 16S rRNA and outer membrane proteins (OMP)-like *LipL41* have been targeted by different research workers to detect pathogenic leptospires by conventional PCR (Rapiphan et al., 2011; Raven, 2006; Senthilkumar et al., 2011). Considering the advantages of each gene over the other, two genes viz., partial *secY* (G1,G2) and *LipL32* were targeted for identification of pathogenic leptospires.

Molecular analysis of the *Leptospira* genome especially nucleotides (nts) sequences of specific genes is paramount significant to determine the genetic variations and the evolutionary relatedness including the understanding of the new species or types evolution. Phylogenetic analysis of the isolates or the PCR amplified products using *rpoB* and *LipL41* genes will give a clear picture on the presence of different species of leptospires, which can not be identified completely by MAT or by other PCR techniques. As per Venkatesha and Ramadass (2001), Bevans et al. (2020), Chandan et al. (2016), *rpoB* gene can be effectively used for phylogenetic analysis and detection of *Leptospira* species from field materials. The outer membrane proteins like *LipL41* plays a crucial role in obligatory bacteria like leptospires and sequence variability can be ascertained effectively in OMPs (Lascola et al., 2006; Marjo and Windell, 2019). The phylogenetic analysis will also help us to understand the prevalence of *Leptospira* intermediate species which are playing a very significant role in the disease control strategy in India as per the earlier findings (Balamurugan et al., 2013). The phylogenetic analysis targeting two different genes will further help us to know the molecular epidemiology of *Leptospira* species in the geographical area.

## 2. Methods

**Collection of samples:** Cows and Buffaloes with clinical signs and history of suspected leptospiral infections like abortion, still-birth, infertility problems (repeat breeder, pyometra and cystic ovaries), blood in milk and icterus with dullness, haematuria were chosen for study purpose and samples like blood, serum, urine, milk, aborted materials and tissue samples were collected from these animals.

**Isolation of leptospires:** All the samples collected from MAT positive animals were used for isolation i.e., sediments in liquid samples and liquid suspension just above the sediment for tissue samples (3–4 drops) were inoculated into the liquid EMJH media using 0.45 µm syringe filter. The tubes were incubated at 30 °C

for 4–6 weeks with a weekly observation and sub culturing as per the requirement.

### 2.1. Polymerase chain reaction (PCR)

**DNA extraction:** The DNA extraction from the suspected materials was carried out using QIAmp DNA mini kit – (Qiagen, Cat. No: 51306) following the manufacturer's instructions.

**Primers:** The following sets of primers were used for the gene G1 F – 5' CTG AAT CGC TGT ATA AAA GT 3' and G2 R – 5' GGA AAA CAA ATG GTC GGA AG-3' [4]. *LipL32-F* 5' CATATGGGTCTGC-CAAGCCTAAA 3' and *LipL32-R* 5' CTCGAGTTACTTAGTCGCGTCAGAA 3' [3]. The PCR reaction was done in 25 µl as previously described (Bal et al., 1994; Meenambigai et al., 2011). The PCR was performed in the total volume of 25 µl reaction mixture as follows:

Master Mix 12.5 µl, Forward Primer (10 pmole/µl) 1.0 µl, Reverse Primer (10 pmole /µl) 1.0 µl, Template DNA 4.0 µl, Nuclease free water 6.5 µl, Total 25.0 µl, For positive control four µl of DNA template (*L. interrogans* serovar Hardjo) and for Negative control known negative DNA (*Staphylococcus* species) was used.

For *secY* PCR, using primer G1 and G2, one cycle of 94 °C for 5 min for initial denaturation followed by 32 cycles of 94 °C for 1 min-denaturation, 55 °C for 1 min- Annealing, 72 °C for 2 min for extension and final extension of one additional cycle of 72 °C for 6 min.

**Agarose gel electrophoresis:**

Following electrophoresis, the gel was visualized at 300 nm wave length using a UV transilluminator for the presence of specific amplicons along with DNA molecular weight marker.

### 2.2. Phylogenetic analysis

The PCR positive samples and the isolates were amplified for sequencing and subsequent phylogenetic analysis using partial *rpoB* and *LipL41* gene sequences using the primers *rpoB-F*: 5'CTCATGGGTCCCAACATGCA 3' and *rpoB-R* :5'CGCATCTCRAAG TTGTAWCCTT 3' and *LipL41-F* 5' TAGGAAATTGCGCAGCTACA 3' and *LipL41-R*: 5'GCATCGAGAGGAATTAACATCA 3' and PCR conditions as described earlier (Balamurugan et al., 2013; Bal et al., 1994; Ahmed et al., 2006) along with positive and negative controls. For partial *rpoB* first cycle of 94 °C for 3 min for initial denaturation, followed by 39 cycles of 94 °C for 90 sec denaturation, 55 °C for 1 min Annealing, 72 °C for 45 sec for extension and final extension of final extension of 72 °C for 20 min. Similarly, for *LipL41*, first cycle 95 °C for 5 min for initial denaturation and then 34 cycles of 94 °C for 30 sec denaturation, 58 °C for 30 sec Annealing, 72 °C for 1 min for extension and final extension of one cycle of 72 °C for 6 min.

The amplicons were commercially sequenced and edited nts sequences by mMEGA 5 software was analyzed by BLAST and Clustal W methodology using the published sequences (Tables 1 and 2).

## 3. Results and discussion

### 3.1. Isolation of leptospires in EMJH medium

The different samples collected from the MAT positive animals were subjected for isolation, out of which three samples (1.84%) yielded growth of leptospires (Fig. 1) during the second week of incubation. Eventhough, 12 samples were positive by PCR, we could isolate leptospires only from three samples (25%) indicating that less sensitivity of the isolation method or it may be due to the contamination of the other organism while employing the isolation. However, inspite of repeated sub culture and following strict

**Table 1**Details of the *rpoB* nucleotide sequences of *Leptospira* from different countries available in the GenBank were included for the comparison.

Sl. No.	Place of Origin	GenBank accession no.	Sl.No.	Place of Origin	GenBank accession no.
1	India	JN388642	21	India	HM046990
2	India	JN388657	22	Brazil	EU747301
3	India	HM046991	23	India	HM046995
4	India	JN388617	24	India	HM046989
5	India	JN388655	25	Brazil	EU747315
6	India	HM046992	26	France	DQ296139
7	India	HM046993	27	India	HM046994
8	India	JN388654	28	India	HM046996
9	India	JN388632	29	India	HM046997
10	India	JN388656	30	India	JN388636
11	India	JN388629	31	India	JN388633
12	Brazil	EU747299	32	India	JN388625
13	Brazil	EU747311	33	France	DQ296138
14	Brazil	EU747304	34	France	DQ296143
15	France	DQ296133	35	France	DQ296136
16	India	JN388624	36	France	EU747316
17	India	JN388631	37	France	DQ296142
18	Brazil	EU747310	38	Brazil	EU747309
19	India	JN388644	39	Brazil	EU 747,303
20	Brazil	EU747307	40	India	EU388638

**Table 2**Details of the *LipL41* nucleotide sequences of *Leptospira* from different countries available in the GenBank were included for the comparison.

Sl. No.	Place of Origin	GenBank accession no.	Sl. No.	Place of Origin	GenBank accession no.
1	China	AY622686	21	China	AY776298
2	Brazil	GQ204270	22	USA	AY461952
3	India	DQ132992	23	Mayotte	JN683923
4	USA	AY461945	24	USA	AY461957
5	USA	AY461946	25	USA	AY461956
6	Argentina	KF184576	26	USA	AY461950
7	China	AY622673	27	USA	JN461949
8	China	AY622677	28	India	JN683923
9	China	AY622679	29	USA	AY461953
10	Japan	AY240677	30	USA	AY461954
11	India	AY642286	31	USA	AY461958
12	China	AY622687	32	China	AY622674
13	China	AY622678	33	China	AY622676
14	Japan	AB240674	34	China	AY622684
15	China	AY622683	35	USA	AY461937
16	China	AY776300	36	Mayotte	JN683912
17	China	AY776299	37	Mayotte	JN683918
18	Japan	AB240676	38	Japan	AB240677
19	India	AY642287	39	USA	AY461947
20	China	AY622682			

biosecurity and sterility measures the growth was observed in only three samples.

### 3.2. Polymerase Chain reaction (PCR)

The different samples collected from MAT positive animals were initially screened using G1 and G2 primers (Gravekamp et al., 1993) could detect 12 (7.36%) leptospiral DNA with an amplicon size of 285 bp. Subsequently, when all the samples were subjected to *LipL32* specific PCR the same 12 samples were found positive with an amplicon size of 756 bp (Figs. 2 and 3). The identification of *Leptospira* is a fool-proof mechanism to determine the active infection in any given animal, hence genomic detection by PCR was employed to confirm the disease in the seropositive animals as PCR being more specific, rapid, less cumbersome. The detection of same 12 samples as positive by both primer sets has shown the efficacy of both the primer sets and the results were in agreement (Cheema et al., 2007), as they also reported that the above primers were similar in their efficacy for diagnosis of leptospirosis. Further, it was found that all the 12 samples were positive by both primers indicating the absence of Grippotyphosa serogroup/serovar. Moreover, all the PCR positive cases were from

the animals with aborted history especially from various tissues of the aborted foetus has resulted in specific amplicons. This finding clearly indicates that targeting more aborted animals with repeated collections could help in finding more positive cases by PCR.

### 3.3. Nucleotide sequencing of PCR products

Direct sequencing of the purified PCR amplified partial *rpoB* and *LipL41* gene products (Figs. 4, 5) was done using the gene specific primer pairs. The partial *rpoB* gene primer included Lept 1900F and Lept 2500R bordering a hypervariable region between the position 1900 and 2500. The *LipL41* gene primer included the polymorphic sequence size of 518 coating for outer membrane lipoprotein with a PCR product size of 520 bp (Ahmed et al., 2006). There is an exponentially high sequencing data of biological genomes is available now for molecular biological or genetic investigations (Bansal, 2005). The genetic analysis of leptospires is to be established to show the genetic relatedness and evolutionary variation among the different *Leptospira* species prevalent in a geographical area. The study of nucleotide sequence is important to understand the evolution of new types or species, its importance in the devel-

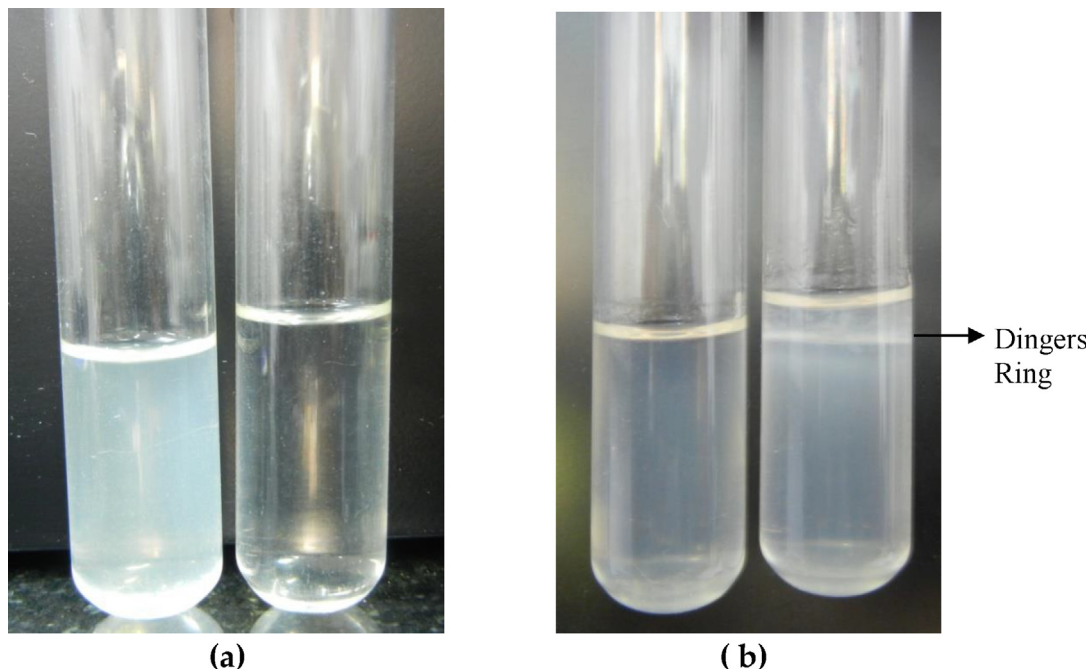


Fig. 1. Growth of leptospires in EMJH liquid (a) growth & control and EMJH semisolid medium (b) control & growth.

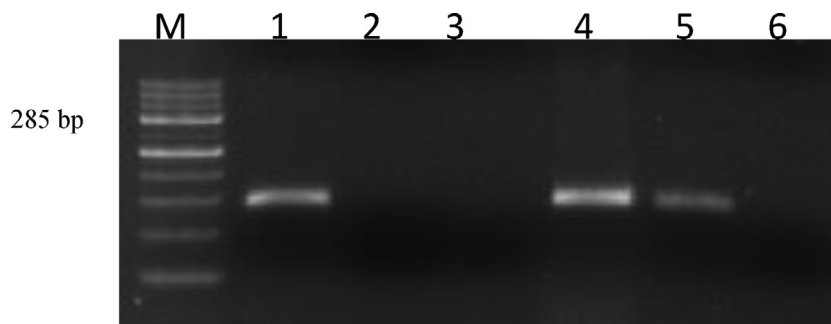


Fig. 2. Agarose gel showing PCR amplifications with G1G2 Primers. Lane M: 100 bp ladder; Lane 1: Positive control (Reference *sejroe* strain); Lane 2: Negative control (*Staphylococcus* Spp); Lane 3: No template control; Lane 4: Positive isolate (KVAFSU\_BANG\_KAR I); Lane 5: Positive isolate (KVAFSU\_BANG\_KAR II); Lane 6: Negative sample.

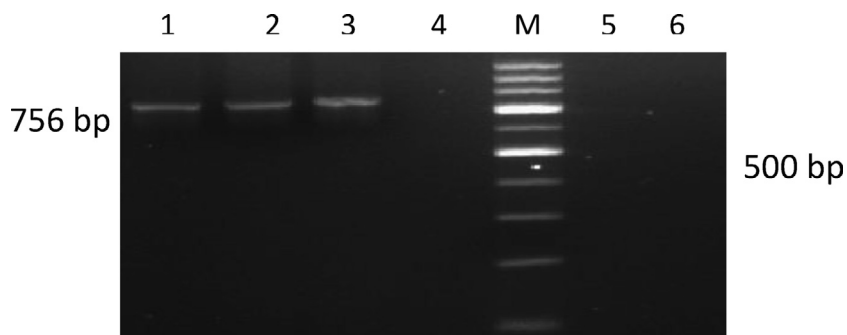
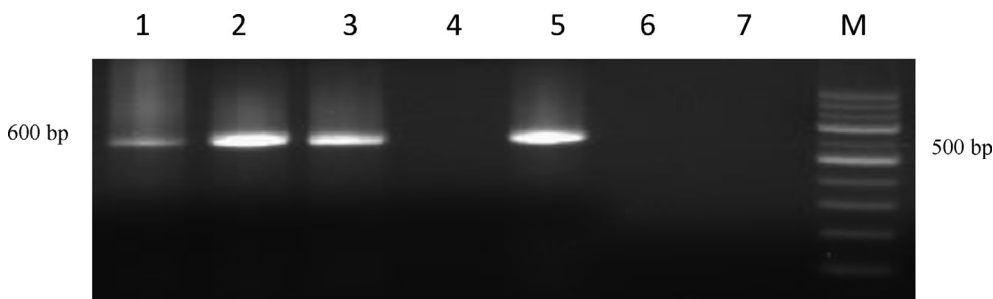


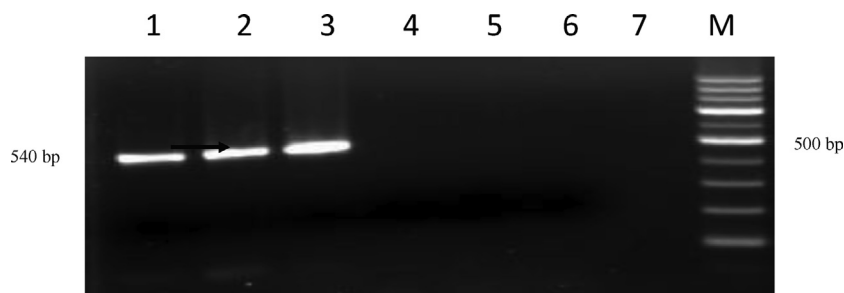
Fig. 3. Agarose gel showing PCR amplifications with *LipL32* Primers. Lane M: 100 bp ladder; Lane 1: Positive sample (KVAFSU\_BANG\_KAR 1); Lane 2: Positive isolate (KVAFSU\_BANG\_KAR I); Lane 3: Positive control (Reference *sejroe* strain); Lane 4: Negative control (*Staphylococcus* Spp); Lane 5: Negative template control; Lane 6: Negative sample.

opment of diagnostic and molecular epidemiological investigations. Further, this technique is beneficial in identification of even single nucleotide deletion / addition or substitution, which cannot be detected in any other methods.

In principle, the phylogenetic analysis based on alignment of nucleotide sequence of specific genes provide an insight into the genetic makeup of the leptospires and their sharing with the other members of the group. This approach is perhaps the most realistic



**Fig. 4.** Agarose gel showing PCR amplification with *rpoB* Primers. Lane M: 100 bp ladder; Lane 1: Positive isolate (KVAFSU\_BANG\_KAR I); Lane 2: Positive isolate (KVAFSU\_BANG\_KAR II); Lane 3: Positive isolate (KVAFSU\_BANG\_KAR III); Lane 4: Negative control (*Staphylococcus* Spp); Lane 5: Positive control (Reference *canicola* strain); Lane 6: No template control; Lane 7: Negative sample.



**Fig. 5.** Agarose gel showing PCR amplification with *LipL41* Primers Lane M:100 bp ladder; Lane 1: Positive isolate (KVAFSU\_BANG\_KAR I); Lane 2: Positive isolate (KVAFSU\_BANG\_KAR II); Lane 3: Positive control (Reference *canicola* strain); Lane 4: Negative control (*Staphylococcus* Spp); Lane 5: No template control; Lane 6 and 7: Negative sample.

and presents a quick bird's eye view of the complex inter-relatedness.

### 3.4. Phylogenetic analysis

The Phylogenetic trees were constructed and the evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1997) using corresponding sequences from both published reference *rpoB* and *LipL41* sequences from different parts of the world and nucleotide sequences obtained in this study. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Neighbour-Joining tree method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved nucleotide sequences from reference strains as well as amplified products sequenced in this study. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2007) The details are shown as per the cluster formation in (Figs. 6, 7).

#### 3.4.1. Analysis of partial *rpoB* gene sequences of the *Leptospira*

Several molecular techniques have been evaluated for the identification and characterization of *Leptospira* species and DNA-DNA hybridization identified 20 *Leptospira* species to date with nine pathogenic *Leptospira* species (Cerqueira and Picardeau, 2009). Analysis of a segment of *rpoB* may be useful as an initial screening test for the identification of a new isolate of *Leptospira* using a system of similarity cut-off to define species (Lascola et al., 2006).

Out of the 12 PCR positive samples, 11 samples gave a reasonably good sequence read sufficient enough for further analysis when subjected to nucleotide sequencing for partial *rpoB* gene (Fig. 6). By sequence analysis, the positive samples showed identity

with the many published sequences of various leptospire of NIVEDI, Bangalore (formerly PD\_ADMAS), India and other global isolates. In general, all the samples have shown identity with *L. interrogans*/*L. borgpetersenii* species by forming various clusters in the same species group. None of the samples have formed cluster with *Leptospira krischneri* and *Leptospira intermediate* species as well as *Leptospira biflexa* and few other serovars / species which were used as out group to compare the sequences.

When the above 11 amplified products were compared within the *L. interrogans*/*L. borgpetersenii* species, it was clearly evident that they formed three different clusters i.e., KVAFSU\_BANG\_KAR 1–6 have formed one cluster and KVAFSU\_BANG\_KAR 7–9 have formed another cluster. KVAFSU\_BANG\_KAR 10 and 11 have formed a different cluster. The cluster formation was almost matching with the findings of multiplex PCR (mPCR) where the samples KVAFSU\_BANG\_KAR 1–4 have shown amplification with *Leptospira interrogans* serovar *canicola* and in the cluster formation also the above four samples have shown clear identity with the *L. interrogans* serovar *canicola*. Similarly, KVAFSU\_BANG\_KAR 7–9 has shown amplification with *Leptospira interrogans* serovar *sejroe* and in the cluster formation also the above three samples have shown identity with the *L. interrogans* serovar Hardjo. KVAFSU\_BANG\_KAR 10 and 11 has shown amplification with *L. interrogans* serovar Icterohaemorrhagiae and in the cluster formation also above two samples have shown identity with the *L. interrogans* serovar Icterohaemorrhagiae. For samples 5 and 6 even though they have shown identity with serovars *Canicola*, *Hebdomadis* and few other isolates but based on mPCR and nucleotide sequencing of partial *rpoB* findings it can be concluded that they belong to other serovar of *L. interrogans*/*L. borgpetersenii* species.

The present findings were in agreement with the findings of (Lascola et al., 2006) and (Bal et al., 1994) they also stated that partial *rpoB* gene can be effectively used for phylogenetic analysis, as

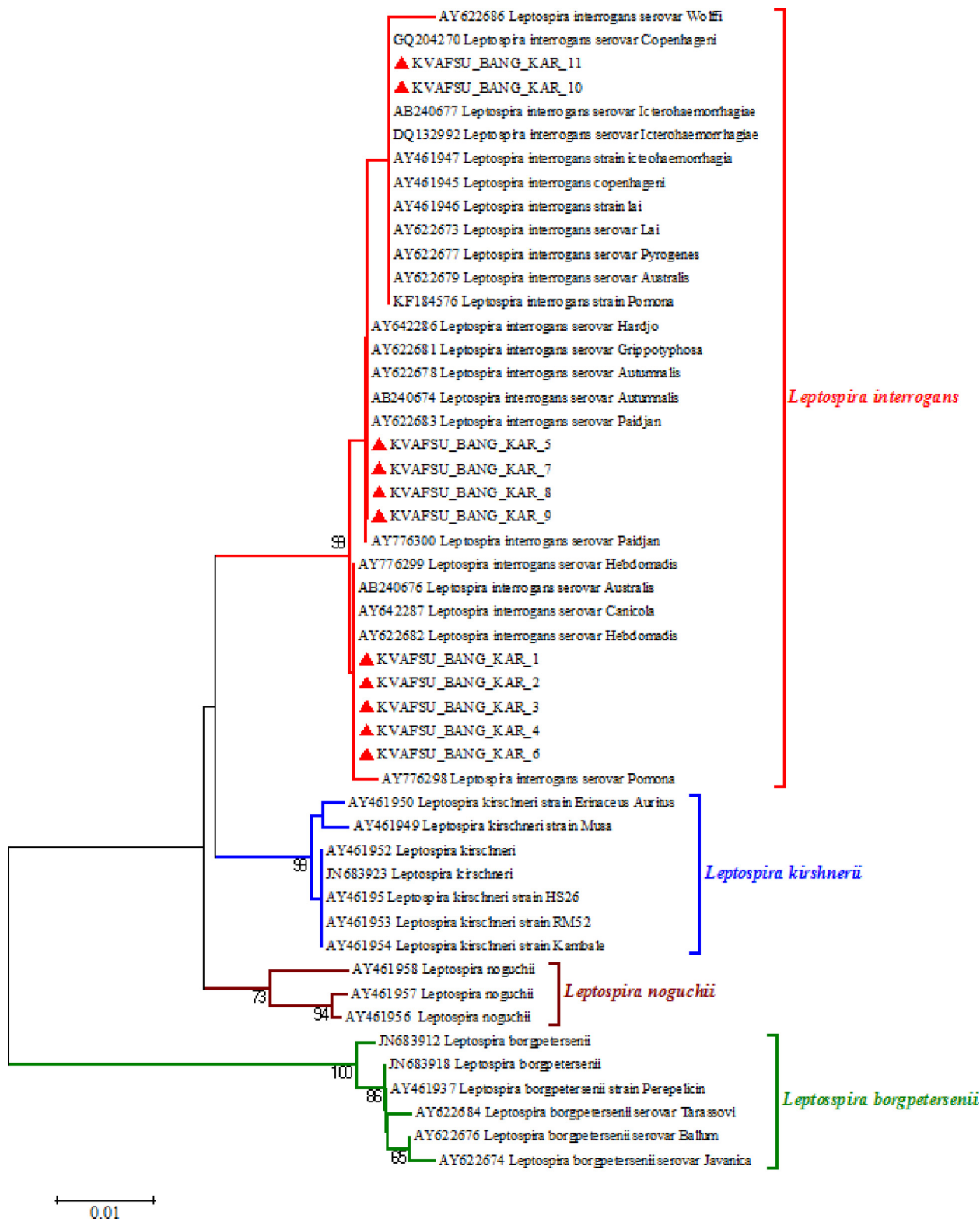


Fig. 6. Phylogenetic tree of partial *rpoB* gene nucleotide sequences using neighbour-joining method.

well as identification of leptospires from clinical samples (Bal et al., 1994) in their findings clearly stated that 30.3% of *Leptospira* isolates isolated from different parts of India belong to *L.interrogans*/*L.borgpetersenii* species and in the present study also all the 11 PCR amplified products were belonging to the above species group and none of them were belonging to *L.intermediate* group.

### 3.4.2. Analysis of partial *LipL41* gene sequences of the *Leptospira*

Out of the 12 PCR positive samples, 11 samples gave a reasonably good sequence read sufficient enough for further analysis when subjected to nucleotide sequencing for *LipL41* gene (Fig. 7). The *LipL41* gene includes the polymorphic sequence size of 518 coating for outer membrane lipoprotein with a PCR product size

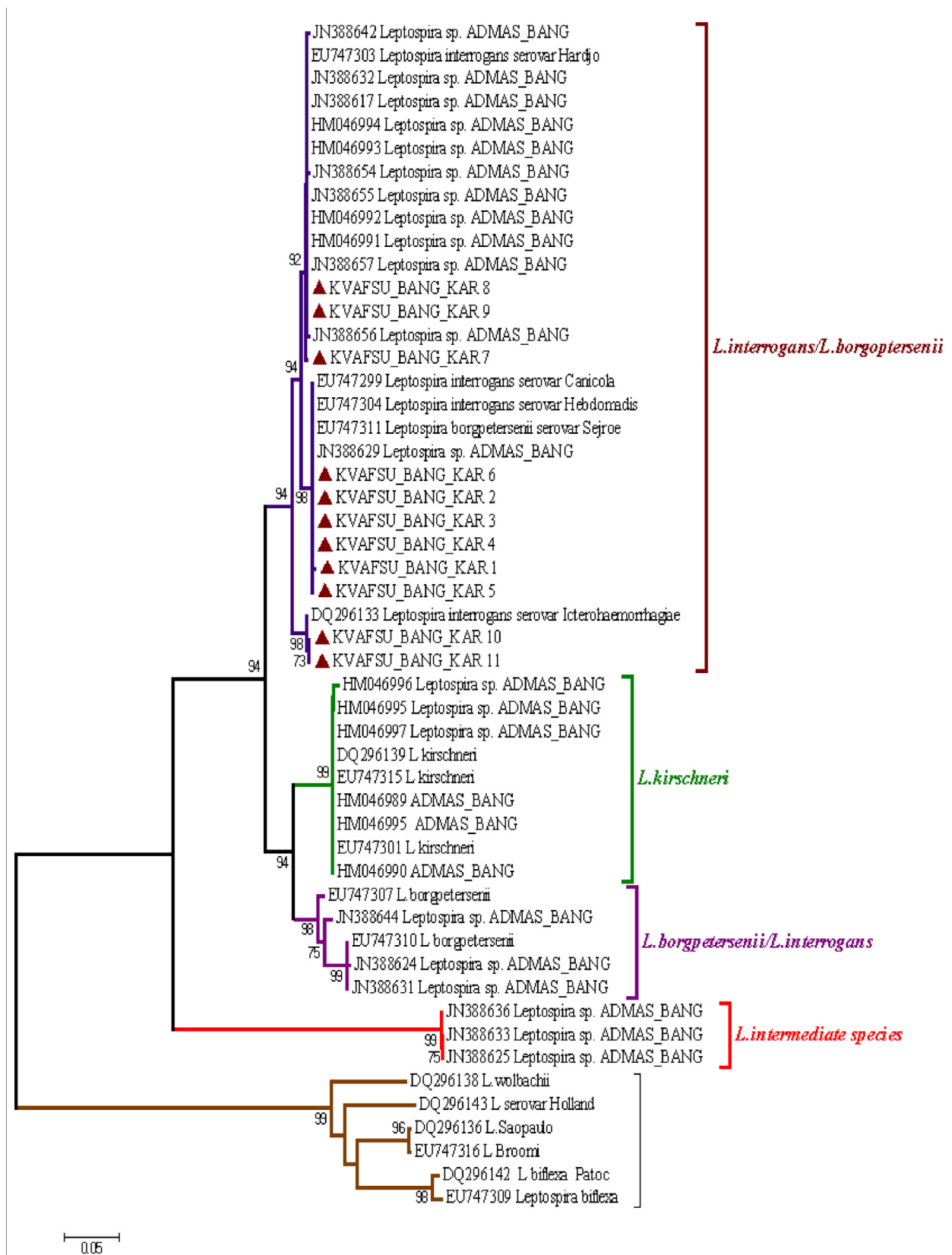


Fig. 7. Phylogenetic tree of LipL41 gene nucleotide sequences of *Leptospira* constructed based on neighbour-joining method.

of 520 bp (Ahmed et al., 2006). In obligate intracellular bacteria, the outer membrane proteins (OMPs) play a crucial role in the process of adaptation by facilitating interactions between bacterial cells and its host (Nguyen et al., 2006).

Though more than 258 serovars in the genus *Leptospira* have been identified, they are antigenically distinct due to the lipopolysaccharide (LPS) and spatial arrangement of outer membrane lipoproteins (OmpL). Generally, the 16S rRNA genes are most

conserved but sequence variability is noticed in outer membrane proteins (Vedhagiri et al., 2009). Keeping these points in view to identify the speciation of the leptospires and evolutionary changes *LipL41* was targeted in the present study using the published nucleotide sequences.

Most of the published sequences belong to human and less data was available pertaining to animals. This was probably the first study to compare the animal isolates or samples to compare with the published human isolates for the *LipL41* gene region in India. Interestingly, unlike with *rpoB* gene region, all the sequenced amplified products shown the identity with the *L.interrogans* species and there was no overlapping of *L.interrogans/L.borgpetersenii* species observed. When the above 11 amplified products were compared within the *L.interrogans* species it was clearly evident that they formed three different clusters i.e., KVAFSU\_BANG\_KAR\_1–4 and 5 have formed one cluster and KVAFSU\_BANG\_KAR\_6 and 7–9 have formed another cluster. KVAFSU\_BANG\_KAR\_10 and 11 have formed a different cluster. The cluster formation was almost matching with the findings of mPCR where the samples KVAFSU\_BANG\_KAR\_1–4 have shown amplification with *L. interrogans* serovar Canicola and in the cluster formation also the above four samples have shown clear identity with the *L.interrogans* serovar Canicola. Similarly, KVAFSU\_BANG\_KAR\_7–9 has shown amplification with *L.interrogans* serovar Hardjo and in the cluster formation also the above three samples have shown clear identity with the *L.interrogans* serovar Hardjo. KVAFSU\_BANG\_KAR\_10 and 11 has shown amplification with *L.interrogans* serovar Icterohaemorrhagiae and in the cluster formation also above two samples has shown clear identity with the *L.interrogans* serovar Icterohaemorrhagiae. For sample 5 even though it has shown more identity with serovars Autumnalis, Hardjo, Paidjan and Grippotyphosa and few other isolates but based on mPCR and nucleotide sequencing of *LipL41* findings, it was concluded that it belongs to other serovars of *L.interrogans* species. For sample 6 even though it has shown more identity with serovars Hebdomadis, Canicola, Australis and Pomona but based on multiplex PCR and nucleotide sequencing of *LipL41* findings, it was concluded that it belongs to other serovar of *L.interrogans* species.

The observations were in agreement with the other's findings (Vedhagiri et al., 2009), in their study also *L.interrogans* has formed a separate cluster when compared with the *L.borgpetersenii* species. Apart from this the present observation of forming a single cluster of *L.interrogans* species was in agreement with the findings of previous report, where the *LipL41* gene has highly conserved sequences that are expressed both in cultivated organisms and during infection in mammals (Haake et al., 1999). Furthermore, the present finding also manifest that the PCR positive samples have indicated same result with respect to conserved sequence during the infection.

#### 4. Conclusion

Interestingly, results analysis of both *rpoB* and *LipL41* genes were compared with the species differentiation, which could not be done completely when *rpoB* gene sequences alone was targeted, as in that there was an overlapping/superimposition between the *L.interrogans/L.borgpetersenii* species was observed as reported earlier. It is noteworthy that these findings in our present work clearly pointed out that *LipL41* can be more efficiently used for species identification of leptospires when compared to *rpoB* gene sequences alone, but this needs to be ascertained on a larger number of samples from different geographical areas of the endemic region.

#### Funding

The study was funded by IAH&VB, Hebbal, Bengaluru, India.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Acknowledgements

Authors thank the Director, IAHVB and VC, KVAFSU for providing necessary funding and guidance throughout the study. The authors extend their appreciation to the Researchers Supporting Project Number (RSP-2020/201), King Saud University, Riyadh, Saudi Arabia.

#### References

- Hartskeerl, P.A., Terpstra, W.J., 1996. Leptospirosis in wild animals. *Vet. Q.* 18, 149–150. <https://doi.org/10.1080/01652176.1996.9694722>.
- World Health Organization, 1999. Leptospirosis worldwide. *Wkly. Epidemiol Rec.* 74, 237–242.
- Levett, P.N., 2011. Leptospirosis. *Clin. Microbiol. Rev.* 14, 296–326.
- Dhaliwal, G.S., Murray, R.D.E., WA.1996. Reproductive performance of dairy herds infected with *Leptospira interrogans* serovars hardjo relative to the year of diagnosis. *Vet. Rec.* 138, 272–276.
- Guitian, F.J., Thurmond, M.C., Hietala, F.K., FK.1999. Infertility and abortion among first lactation on dairy cows seropositive or seronegative to *Leptospira interrogans* serovars hardjo infection. *J. Am. Vet. Med. Assoc.* 215, 515–518.
- Rapiphan, U., Supajit, S., Duangjai, S., Kanokporn, T., 2011. PCR based technique for detection and differentiation of Pathogenic and Saprophytic *Leptospira* species. *Intl. J. Microbiol. Res.* 2, 43–48.
- Raven, ER.2006. Development of species-specific PCR primer sets for the detection of *Leptospira*. *FEMS Microbiol. Lett.* 264, 31–39.
- Senthilkumar, A., Ramadass, P., Nachimutu, K., 2011. Use of polymerase chain reaction for the detection of leptospires in clinical samples. *Indian Vet. J.* 78, 1087–1090.
- Venkatesha, M.D., Ramadass, P., 2001. Rapid diagnosis of leptospirosis using PCR and DNA hybridization techniques. *Indian J. Anim. Sci.* 72, 1104–1106.
- Bevans, A.I., Fitzpatrick, D.M., Stone, D.M., Butler, B.P., Smith, M.P., Cheetam, S., 2020. Phylogenetic relationship and diversity of bat associated *Leptospira* and the histopathological evaluation of these infections in bats from Grenade, West Indies. *PLoS. Negl. Trop. Dis.* 2014,1–15.
- Chandan, S., Sharanaiyah, U., Govindaraju, S., Vinayaka, B., 2016. Use of *rpoB* gene analysis for detection and identification of *Leptospira* species by direct sequencing. *Eur. J. Biotech. Biosci.* 4, 34–43.
- Lascola, B.L., Bui, L.T.M., Baranton, G., Khamis, A., Raoult, D., 2006. Partial *rpoB* gene sequencing for identification of *Leptospira* species. *FEMS. Microbiol. Lett.* 263, 142–147.
- Marjo, V.M., Windell, LR.2019. Identification of *Leptospira* sps from environmental sources in areas with high human leptospirosis incidence in the Philippines. *Pathogen and Global Health* 113, 109–116.
- Balamurugan, V., Gangadhar, N.L., Mohandoss, N., Thirumalesh, S.R.A., Dhar, M., Shome, R., Krishnamoorthy, P., Prabhudas, K., Rahman, H.2013. Characterization of *Leptospira* isolates from animals and humans: phylogenetic analysis identifies the prevalence of intermediate species in India. *Springer. Plus.* 2, 362.
- Bal, A., Gravekamp, E.C., Hartskeerl, R.A., Brewster, J.D.M., Korver, H., Terpstra, W.J.1994. Detection of leptospires in urine by PCR for early diagnosis of leptospirosis. *J. Clin. Microb.* 32, 1894–1898.
- Meenambigai, T.V., Gopalakrishnan. Ravikimar. Andy, S., Govindan, B., Chidambaram, S., Bakthavachalam, M., 2011. Simultaneous detection of *LipL32* and *LipL21* genes of Pathogenic *Leptospira* from serum samples of bovines by multiplex PCR. *Vet. Sci Dev.* 1, 60–62.
- Ahmed, N., Devi, S.M., Valverde, M.L., Vijayachari, P., Machangus, R.S., 2006. Multilocus sequence typing method for identification and genotypic classification of pathogenic *Leptospira* isolates. *PLoS One.* 5, 1533.
- Gravekamp, C., VandkhFranzen, M., Carrington, D., Schoone, G.J., Vaneys, G.J.J.M., Everard, C.O.R., Hartskeerl, R.A., Terpstra, W.J.1993. Detection of seven species of pathogenic leptospires by PCR using two sets of primers. *J. Gen. Microbiol.* 139, 1691–1700.
- Cheema, P.S., Srivastava, S.K., Amutha, R., Singh, S., Singh, H., Sandey, M., 2007. Detection of pathogenic leptospires in animals by PCR based on *LipL21* and *LipL32* genes. *Indian. J. Expt. Biol.* 45, 568–573.
- Bansal, A.K., 2005. Bioinformatics in microbial biotechnology: A mini review. *J. Micro. Cell. Fact.* 4, 19.
- Saitou, N., Nei, M., 1987. The neighbour-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425.
- Felsenstein, J., 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39, 783–791.
- Tamura, K., Nei, M., Kumar, S., 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Nat. Acad. Sci.* 10, 11030–11035.



- Tamura, K., Daniel, P., Nicholas, P., Glen, S., Masatoshi, N., Kumar, S., 2007. MEGA 5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739.
- Cerqueira, G.M., Picardeau, M., 2009. A century of *Leptospira* strain typing. *Infect. Genet. Evol.* 9, 760–768.
- Nguyen, T.X., Alegre, E.R., Kelley, S.T., ST.2006.. Phylogenetic analysis of general bacterial porins: a phylogenomic case study. *J. Mol. Microbiol. Biotechnol.* 11, 291–301.
- Vedhagiri, K., Natarajaseenivasan, K., Cheellapandi, P., Prabhakaran, S.G., Selvin, J., Sharma, S., Vijayachari, P., 2009. Evolutionary implication of outer membrane lipoprotein-encoding genes *ompL1*, *lipL32* and *lipL41* of pathogenic *leptospira* species. *Genomics. Proteomics. Bioinf.* 7, 96–106.
- Haake, D.A., Walker, E.M., Blanco, D.R., Bolin, C.A., Miller, M.N., Lovett, MA.1999.. Changes in the surface of *Leptospira interrogans* serovar grippityphosa during invitro cultivation. *Infect. Immun.* 59, 1131–1140.