RESEARCH ARTICLE
Open Access

Journal of Veterinary and Animal Sciences
ISSN (Print): 0971-0701, (Online): 2582-0605
https://doi.org/10.51966/jvas.2021.52.3.238-244

Multi-locus sequence typing for species/ serovar identification of clinical isolates of *Leptospira* spp.*

D. Divya[1], Siju Joseph[2], M. Mini[3], R. Sreeja Nair[4] and K. Justin Davis[5]**
Department of Veterinary Microbiology,
College of Veterinary and Animal Sciences, Mannuthy, Thrissur- 680 651
Kerala Veterinary and Animal Sciences University, Kerala, India.

Citation: Divya, D., Siju Joseph., Mini, M., Sreeja Nair, R. and Justin Davis, K. 2021. Multi-locus sequence typing for species/serovar identification of clinical isolates of *Leptospira. J. Vet. Anim. Sci.* **52**(3): 238-244. DOI: https://doi.org/10.51966/jvas.2021.52.3.238-244

Received: 18.01.2021 *Accepted: 08.03.2021* *Published: 30.09.2021*

Abstract

Leptospirosis is an emerging zoonotic disease endemic in Kerala and close monitoring of emerging serovars is essential to adopt appropriate control strategies. Multi-Locus Sequence Typing (MLST) was reported to be less expensive compared to other cumbersome methods like whole genome sequencing. The present study was conducted to obtain isolates of Leptospira from infected animals and rats and for the identification of serovars using MLST. A total of 205 blood samples (dog, cat, cattle, goat), 43 urine samples (dog, cattle) and post-mortem kidney samples from various animals such as dog (n=12), cattle (n=2) and rat (n=25) were collected and subjected to polymerase chain reaction (PCR) using G1/G2 primers to identify the pathogenic Leptospira. Fifteen samples were found to be positive, these samples when inoculated in the Ellinghausen-McCullough-Johnson-Harris (EMJH) semi-solid medium to obtain ten isolates. These ten isolates were further subjected to secY, icdA and GyraseB PCR and sequenced. The obtained sequences were analysed using BLAST and were fed into specified MLST database of Leptospira scheme-3, the allelic profile and sequence type were generated. The MLST results obtained in the study indicated that the isolates S24 and S33 belonged to serovar Canicola, S40 and 47 were Sejroe and S19, S27, S55, S69 and S71 were Bataviae, Autumnalis, Pomona, Icterohaemorraghiae and Australis, respectively. It was concluded that MLST is a convenient method for identifying leptospiral serovars.

Keywords: *Leptospirosis, isolation, PCR, MLST.*

**Part of M.V.Sc thesis submitted by the first author to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala*

1. *M.V.Sc Scholar*
2. *Associate Professor*
3. *Professor and Head*
4. *Assistant Professor*
5. *Assistant Professor, Department of Veterinary Epidemiology and Preventive Medicine*

***Corresponding author email: divya126dd94@gmail.com, Ph. 8148676243*

Copyright: © 2021 Divya *et al.* This is an open access article distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Leptospirosis, a widespread zoonotic disease, is significant in both developed and developing countries particularly in tropical regions. It is caused by the pathogenic bacteria of the genus *Leptospira*. The disease affects a variety of mammals including dogs, cattle, sheep, goats, pigs, horses and humans. Rodents spread the disease directly through contaminated urine, water, feed and soil (Harskeerl and Terpstra, 1996). Although vaccination has been carried out in dogs in Kerala, it has been observed that even vaccinated dogs are often diagnosed with leptospirosis with non vaccinal serovars (Abhinay *et al*., 2012). Hence, monitoring the emergence of serovars is of great importance in adopting appropriate control strategies for controlling the disease. Methods for monitoring different serovars include Microscopic Agglutination Test (MAT), the gold standard serological test for identification leptospiral serovars. However, for performing MAT, live leptospiral culture should be maintained for use as antigen and technically cumbersome. Hence, alternative DNA based methods such as Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP), Pulsed Field Gel Electrophoresis (PFGE), Variable Number Tandem Repeat (VNTR), and Multi-Locus Sequence Typing (MLST) were used for identification of leptospiral serovars (Ahmed *et al.,* 2006). When compared to all these DNA based methods, MLST was preferred because of its high reproducibility and discriminatory power to differentiate leptospiral serovars (Ahmed *et al*., 2011). Considering these facts, the present study was conducted to isolate leptospires from domestic animals and identify the serovars of the pathogenic *Leptospira* using MLST.

Materials and methods

Sample collection

A total of 205 blood samples were collected from dogs (n=139), cats (n=11), cattle (n=29) and goats (n=26) presented to Teaching Veterinary Clinical Complex, College of Veterinary Animal Sciences, Mannuthy with clinical signs indicating to acute leptospirosis, during the study period of March 2019 to March 2020. Post-mortem kidney tissue from dogs (n=12) and cattle (n=2) received from Department of Veterinary Pathology, CVAS, Mannuthy and those from rats (n=25), trapped from college premises, hostels and farms in Mannuthy campus were used in the study. Urine samples were also collected from dogs (n-40) and cattle (n-3). Whole blood samples collected in heparin and ethylene diamine tetra-acetic acid (EDTA) vials were used for isolation of *Leptospira* and to perform PCR, respectively.

Polymerase Chain Reaction

Genomic DNA was extracted from whole blood, urine and kidney samples using Qiagen DNA extraction kit. The concentration and purity was checked by NanoDrop spectrophotometer and DNA was stored at -20°C. The genomic DNA extracted from samples were subjected to PCR using specific primers G1 (5'-CTGAATCGCTGTATAAAAGT-3') and G2 (5'-GGAAAACAAATGGTCGGAAG-3') for an expected amplified product of 285 bp (Gravekamp *et al*., 1993). A 25 µL reaction containing 12.5 µL 2X PCR master mix, 1 µL of 10 p*M* of each forward and reverse primers, 5 µL of template DNA and 5.5 µL nuclease free water was prepared. The amplification protocol used was: initial denaturation at 94°C for 5 mints followed by 25 cycles of denaturation (94°C for 1 min.), annealing (55°C for 1 min.) and extension (72°C for 45 sec.), final extension was done for 10 min. at 72°C followed by hold at 4°C for infinity.

Isolation

Whole blood collected in heparinised vials, urine and homogenised kidney tissues were inoculated aseptically into Ellinghausen McCullough Johnson Harris (EMJH) semi-solid medium containing 5-Fluorouracil (400 µg/mL) to prevent contamination. The samples were incubated at 30°C for a time period of three months (Ellis and Thiermann, 1986). The growth of leptospires were examined at weekly intervals using dark field microscopy (DFM) and Polymerase Chain Reaction (PCR) using G1/G2 primers.

MLST

Housekeeping genes namely *secY, icdA* and *GyraseB* were selected for MLST analysis. The primers used for amplification of these three genes are listed in table 1.

The PCR amplification was carried out in a volume of 25 µL reaction in 200 µL capacity PCR tubes containing, 12.5 µL 2X PCR master mix, 1 µL of 10 p*M* of each forward and reverse primers, 5 µL of template DNA and 5.5 µL of molecular biology grade nuclease free water. The optimised PCR conditions for different genes are listed (Table 2).

The PCR products were identified by agarose gel electrophoresis followed by visualisation in gel documentation system, for recording the results.

The *secY, icdA* and *GyraseB* gene amplicons from the isolates were sequenced using commercial sequencing service provided by M/s Scigenome Labs, Kochi.

MLST data analysis

MLST alleles were assigned using the optimized *Leptospira* MLST scheme 3 from pubMLST database (https://pubmlst.org/leptospira/). The sequence of each locus was checked in *Leptospira* MLST database for the determination of the allele and to generate the allelic profile / Sequence Type (ST) numbers. Based on the ST numbers, the serovar differentiation of each isolate was done.

Results and discussion

Polymerase chain reaction was an efficient and accurate tool for diagnosis of leptospirosis (Merien *et al*., 1995). Out of the 205 samples collected in the present study, 15 were found to be positive in PCR using G1/G2 primers. Among the 15 PCR positive samples, 10 samples demonstrated a distinct subsurface white ring like discrete zone (Dinger's ring) growth in EMJH semi-solid medium in three weeks to three months of incubation (Fig. 1).

Table 1. Primer sequence used for MLST analysis

Sl. No.	Primers	Sequence	Amplicon size	Reference
1	*secY* F *secY* R	5'-ATGCCGATCATTTTTGCTTC-3' 5'CCGTCCCTTAATTTTAGACTTCTTC-3'	549 bp	Ahmed *et al.* (2006)
2	*icdA* F *icdA* R	5'-GGGACGAGATGACCAGGAT-3' 5'-TTTTTTGAGATCCGCAGCTTT-3'	674 bp	Ahmed *et al.* (2006)
3	*GyraseB F* *GyraseB* R	5'- ACATCCCATGCACAAAGTGA-3' 5'- CGGAAAGACCTGTTGGATGT -3'	236 bp	Slack *et al.* (2006)

Table 2. PCR protocol for the amplification

Step		Temperature	Time
Initial denaturation		94°C	5 min.
35 cycles	Denaturation	94°C	1 min.
	Annealing	58°C♦ 58.5°C♦♦ 62.5°C♦♦♦	1 min.
	Extension	72°C	45 sec.
Final extension		72°C	10 min.

♦ Annealing temperature of *secY* gene

♦♦ Annealing temperature of *icdA* gene

♦♦♦ Annealing temperature of *GyraseB* gene

Table 3. MLST results

Sl. No.	Sample No.	Possible STs	MLST (Possible Serovar)
1	S07	-	-
2	S19	70	Bataviae
3	S24	34	Canicola
4	S27	53	Autumnalis
5	S33	38	Canicola
6	S40	60	Sejroe
7	S47	74	Sejroe
8	S55	58	Pomona
9	S69	2	Icterohaemorrhagiae
10	S71	35	Australis

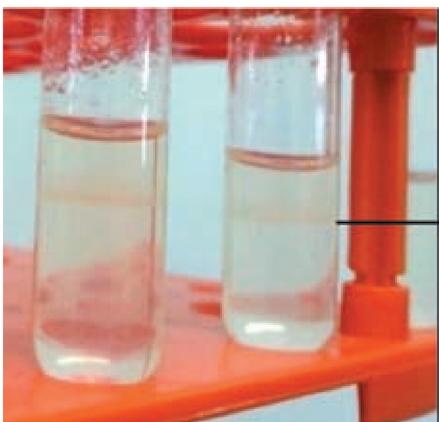

Fig. 1. Growth of leptospires in EMJH semi-solid medium

The isolates were designated as S07, S19, S24, S27, S33, S40, S47, S55, S69 and S71. Among the ten isolates six were from dogs, two from cattle and one each from goats and rats, respectively. Though the percentage of isolation (4.88 per cent) was low in the present study, similar observation was made by Chandran (2017). Reason for low isolation rate could be the smaller number of bacteria in clinical samples owing to the treatment before the sample collection and also sampling which was done to early/late stage of infection.

The isolates obtained were subjected to PCR using G1/G2 primers and observed and all of them yielded 285 bp amplicons (Fig. 2) thus confirming that all were pathogenic *Leptospira*. Similar work has been reported

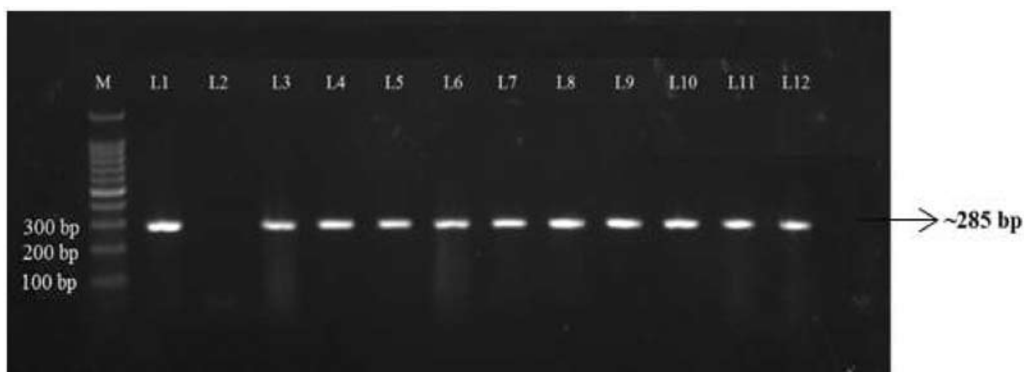

Fig. 2. PCR amplicons from *Leptospira* with G1/G2 primers

Molecular marker	: 100 bp ladder	Lane1	: Positive control
Lane 2	: Negative control	Lanes 3-12	: DNA extracted from the isolates

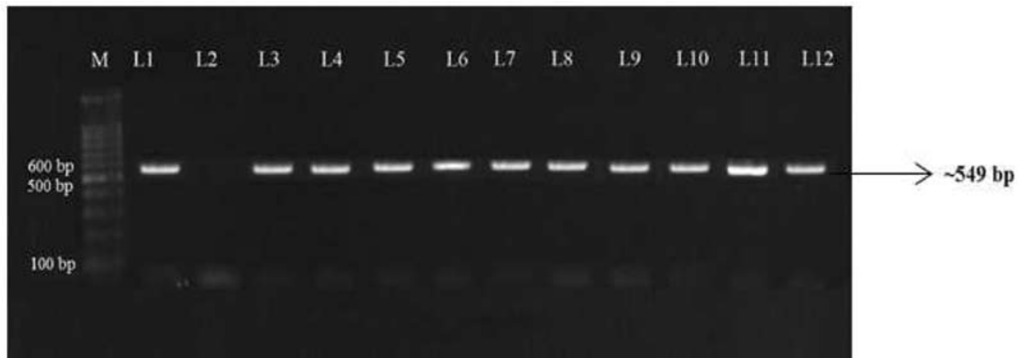

Fig. 3. PCR amplicons of *secY* gene of *Leptospira*

Molecular marker	: 100 bp ladder	Lane 1	: Positive control
Lane 2	: Negative control	Lanes 3-12	: DNA extracted from isolates

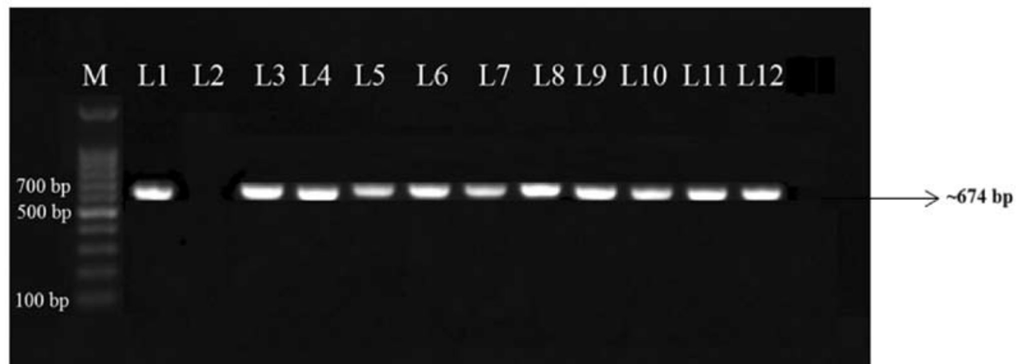

Fig. 4. PCR amplicons of *icdA* gene of *Leptospira*

Molecular marker	: 100 bp ladder	Lane 1	: Positive control
Lane 2	: Negative control	Lanes 3-12	: DNA extracted from isolates

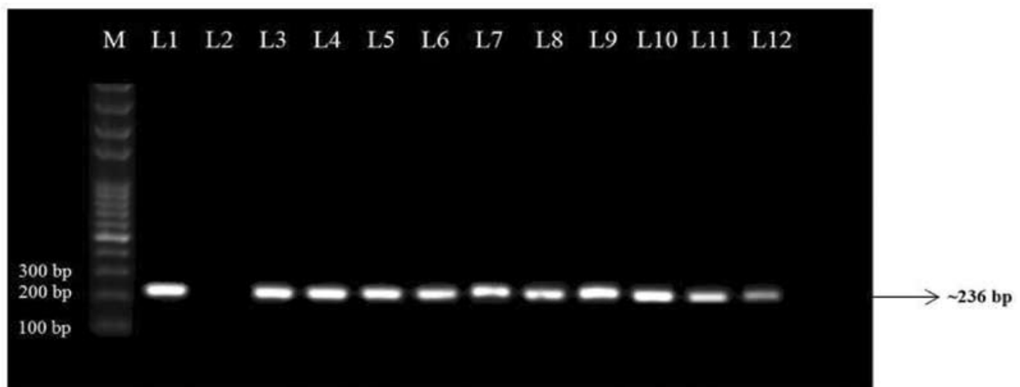

Fig. 5. PCR amplicons of *GyraseB* gene of *Leptospira*

Molecular marker	: 100 bp ladder	Lane 1	: Positive control
Lane 2	: Negative control	Lanes 3-12	: DNA extracted from isolates

by Natarajaseenivasan *et al*. (2011). After confirming the isolates as pathogenic *Leptospira,* they were further subjected to PCR for amplifying *secY, icdA* and *GyraseB* genes, the isolates could successfully yield amplicon size of 549 bp, 674 bp and 236 bp, respectively.

Positive amplicons are depicted in Fig. 3, 4 and 5.

Using optimised *Leptospira* MLST scheme 3 from pubMLST database (https://pubmlst.org/leptospira/), sequence types were obtained as given in table 3. Due to limitations of the study, the present study's focus is on identification of isolates up to the serovar level and not on measuring genetic diversity. Based on Sequence Type number, nine out of ten isolates could be identified at the serovar level (Table 3). The results indicate that the samples S24 and S33 belong to serovar Canicola (Varni *et al*., 2018), S40 and S47 were Sejroe (Bourhy *et al*., 2012), S19, S27, S55, S69 and S71 were Bataviae, Autumnalis, Pomona, Icterohaemorrhagiae and Australis, respectively. Sera samples from the same animals were also subjected to Microscopic Agglutination Test (MAT) and the results were found to be in agreement with the serovars identified using MLST. Remaining one isolate identified by MAT as belonging to serovar Icteroharmorrhagiae failed to generate products for the three genes in the database. The isolate may be further confirmed by advanced typing methods such as PFGE or Whole Genome Sequencing (WGS). A similar issue in identifying the serovar had been reported by Romero *et al*. (2011).

Coclusion

Microscopic agglutination test is routinely performed in reference laboratories for diagnosis and serovar identification. A major drawback is the significant occurrence of cross-reactions between different serovars. Based on the above results, the present study concludes that MLST is a robust method for a more accurate serovar level identification of *Leptospira* which include less cumbersome procedures compared to the more advanced molecular techniques including whole genome sequencing.

Acknowledgement

The authors are thankful to Kerala Veterinary and Animal Sciences University for providing all facilities for the completion of this work.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Abhinay, G., Ambily, R. and Joseph, S. 2012. IgM immunoprofile of leptospiral outer membrane proteins in acute canine leptospirosis. *Indian Vet. J.* **89**: 09-10.

Ahmed, N., Devi, S. M., Valverde, M. de los A., Vijayachari, P., Machang'u, R. S., Ellis, W. A. and Hartskeerl, R. A. 2006. Multilocus sequence typing method for identification and genotypic classification of pathogenic *Leptospira* species. *Ann. Clin. Microbiol. Antimicrob.* **5**: 1-10.

Ahmed, A., Thaipadungpanit, J., Boonsilp, S., Wuthiekanun, V., Nalam, K., Spratt, B. G., Aanensen, D. M., Smythe, L. D., Ahmed, N., Feil, E. J. and Hartskeerl, R. A. 2011. Comparison of two multilocus sequence based genotyping schemes for *Leptospira* species. *PLOS Negl. Trop. Dis.* **11**: 374-376.

Bourhy, P., Collet, L., Lernout, T., Zinini, F., Hartskeerl, R. A., Van der Linden, H., Thiberge, J. M., Diancourt, L., Brisse, S., Giry, C., Pettinelli, F. and Picardeau, M. 2012. Human leptospira isolates circulating in Mayotte (Indian Ocean) have unique serological and molecular features. *J. Clin. Microbiol.* **50**: 307-311.

Chandran, R.A. 2017. Molecular characterization of leptospiral isolates from domestic animals, rats and human beings. *M.V.Sc thesis,* Kerala Veterinary and Animal Sciences University, Pookode, 100 p.

Ellis, W. A. and Thiermann, A. B. 1986. Isolation of leptospires from the genital tracts of Iowa cows. *Am. J. Vet. Res.* **47**: 1694-1696.

Gravekamp, C., van de Kemp, H., Franzen, M., Carrington, D., Schoone, G. J., Van Eys, G. J. J. M., Everard, C. O. R., Hartskeerl, R. A. and Terpstra, W. J. 1993. Detection

of seven species of pathogenic leptospires by PCR using two sets of primers. *Microbiology*. **139**: 1691-1700.

Harskeerl, R. A. and Terpstra, W. J. 1996. Leptospirosis in wild animals. *Vet. Q.* **18**: 149-150.

Merien, F., Baranton, G. and Perolat, P. 1995. Comparison of polymerase chain reaction with microagglutination test and culture for diagnosis of leptospirosis. *J. Infect. Dis*. **172**: 281-285.

Natarajaseenivasan, K., Vedhagiri, K., Sivabalan, V., Prabagaran, S. G., Sukumar, S., Artiushin, S. C. and Timoney, J. F. 2011. Seroprevalence of *Leptospira borgpetersenii* serovar Javanica infection among dairy cattle, rats and humans in the Cauvery river valley of southern India. *Southeast Asian J. Trop. Med. Public Hlth*. **42**: 679-681.

Romero, E. C., Blanco, R. M. and Galloway, R. L. 2011. Analysis of multilocus sequence typing for identification of *Leptospira* spp. isolates in Brazil. *J. Clin. Microbiol*. **11**: 3940-3942.

Slack, A. T., Symonds, M. L., Dohnt, M. F. and Smythe, L. D. 2006. Identification of pathogenic *Leptospira* species by conventional or real-time PCR and sequencing of the DNA gyrase subunit B encoding gene. *BMC Microbiol*. **6**: 1471-2180.

Varni, V., Chiani, Y., Nagel, A., Ruybal, P., Vanasco, N. B. and Caimi, K. 2018. Simplified MLST scheme for direct typing of *Leptospira* human clinical samples. *Pathog. Glob. Hlth*. **4**: 203-209.

■