CANINE LEPTOSPIROSIS: POLYMERASE CHAIN REACTION AS A RAPID ALTERNATIVE TO CONVENTIONAL TECHNIQUES*

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Abstract

Leptospirosis is an acute febrile zoonotic disease with worldwide distribution and has become an important global health concern. The disease is endemic in southern parts of India, especially in Kerala. The laboratory diagnosis of leptospirosis is based on bacteriological, molecular and serological detection methods. The present study was envisaged to compare the molecular techniques for detection of leptospires with the traditional isolation and serological methods. Out of 100 blood samples tested, 27 were found to be positive for microscopic agglutination test (MAT) and from eight samples, leptospires could be isolated successfully in Ellinghausen-McCullogh-Johnson-Harris media (EMJH). MAT positive samples when subjected to polymerase chain reaction (PCR) targeting the lipl32 gene of Leptospira, two samples gave positive amplicons of about 767 bp. Even though, the gold standard test for diagnosis of leptospirosis is isolation and identification of the causative organism, it is laborious, time consuming and requires technical expertise. Thus, the present study recommends the use of MAT for the identification of infecting serovars of Leptospira, while PCR forms an effective tool to provide a rapid diagnosis, particularly in acute infections.

Key words: *Leptospirosis, isolation, Microscopic agglutination test, Polymerase chain reaction, lipl32, EMJH, Canine, Kerala.*

Leptospirosis is a re-emerging zoonosis caused by pathogenic members of the genus *Leptospira* (World Health Organization, 1999). Leptospires are transmitted through infected urine of small mammals, contaminated soil or water, through direct contact with skin lesions or conjunctivae of infected animals (Obiegala *et al*., 2016). It affects both human

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and animals and is mainly characterised by hepatic and renal failure. Leptospirosis is a frequently misdiagnosed disease as many other febrile conditions also elicit similar clinical symptoms. Microscopic agglutination test (MAT) is considered to be the gold standard test among several serological methods for the diagnosis of leptospirosis (Vishak, 2015). Even then, isolation and identification of the organism is definitive and provides information for epidemiological and prophylactic studies. A diagnosis based on isolation techniques and MAT in combination will give ample knowledge about the circulating pathogenic strains within a particular geographical area. Molecular techniques like polymerase chain reaction (PCR) using specific primers targeting the *lipl32* gene, which is conserved in all pathogenic serovars of *Leptospira* not only differentiates pathogenic species from non pathogenic ones, but also serves as a rapid tool for the identification of acute leptospirosis.

Keeping in view of above mentioned facts, the objective of the present study was designed to compare the molecular techniques for detection of leptospires with traditional isolation and serological methods.

Materials and Methods

Attempts for isolation and characterization of the organism were made during the period from February 2016 to March 2017, with samples collected from dogs presented to the University Veterinary Hospitals, Mannuthy and Kokkalai and Kerala Veterinary and Animal Sciences University, Pokoode, Wayanad.

Sample collection

A total of 100 blood and serum samples collected from suspected cases of leptospirosis in dogs. About 3-5 ml of blood was collected aseptically from dogs in sterile heparinized and serum vials for obtaining blood and serum respectively. The samples were submitted to the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy for detailed investigation.

Microscopic agglutination test

The serum samples were screened by MAT using a battery of 10 serovars of *Leptospira interrogans* viz., serovars Australis, Autumnalis, Canicola, Bataviae, Grippotyphosa, Icterohaemorrhagiae, Javanica, Pomona, Patoc and Pyrogenes as per the method described by Krishna (2012).

Isolation

From the collected blood samples, 2-3 drops were inoculated into 5 ml of Ellinghausen-Mc Cullogh-Johnson-Harris media (EMJH) (DIFCO-USA) supplemented with bovine serum albumin supplement (10 per cent) and 5-fluorouracil (200µg/ml) for isolation. The culture tubes were incubated at 28˚-30˚C for a period of up to three weeks to three months and checked weekly under dark field microscope (DFM) before being discarded as negative.

Polymerase chain reaction

DNA was extracted from MAT positive samples using DNA isolation kit (Qiagen, Courtaboeuf, France) following the manufacturer's instructions and samples were stored at -20°C. The PCR assay was carried out with primers targeting *lipl32* gene (Vishak, 2015) to differentiate the pathogenic species. Primers used in the study were enlisted in Table 1. A 12.5µl reaction containing 6.25µl 2X PCR master mix, 1µl of 10 picomoles of each primer, 2µl of template (25ng/µl) and 2.25µl nuclease free water was prepared.

The temperature cycling conditions for PCR were as follows:

Initial denaturation	94°C for 4 min	
Denaturation	94°C for 1 min	35 cycles
Annealing		
Elongation	72°C for 2 min	
Final extension	72°C for 10 min	

Identification of PCR product was done in a submerged agarose gel electrophoresis system using one per cent agarose stained with

ethidium bromide, and Tris Borate EDTA buffer was used as the matrix at a voltage of 50V. The gel was visualised under a UV transilluminator and results were documented in Gel Doc system (Biorad, USA).

Results and Discussion

Out of the 100 samples tested, 27 were found to be positive for MAT. Titres of 1:400 and above were considered as positive for antibodies against *Leptospira* (Vishak, 2015). The results of MAT are depicted in Table 2. The predominant serovars identified were Australis (29 per cent) and Autumnalis (18 per cent) followed by Icterohaemorrhagiae (14 per cent), Bataviae (11 per cent), Pyrogenes (7 per cent), Grippotyphosa (7 per cent), Canicola (3 per cent), Pomona (3 per cent), and Javanica (3 per cent). A similar observation was made by Ambily *et al*. (2012) indicating the predominance of Autumnalis (23.97 per cent) followed by Australis (19.17 per cent) in Thrissur district. The predominance of non vaccinal serovars in dogs raises great concern in lieu of the fact that clinical leptospirosis may occur even in

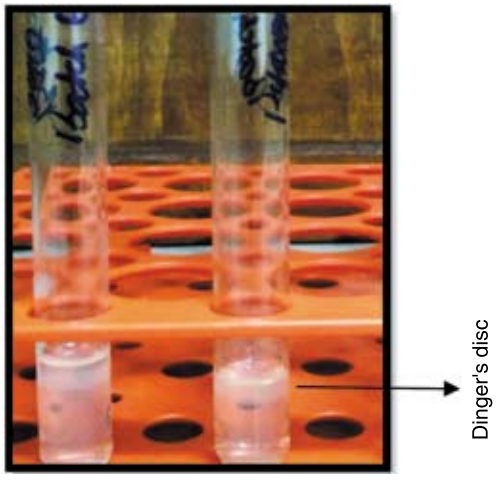

Fig.1: Growth of leptospires in semisolid media

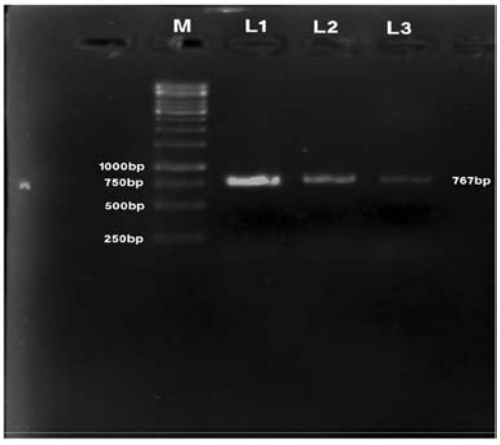

Fig. 2: *Lipl32* gene amplification of isolates - M: 1Kb DNA marker L1: Positive control Lane 2 &3: amplified *lipl32* genes from isolates

Table 1. Primers used used for the amplification of *lipl32* gene *Leptospira*

Primers	Sequence	Amplicon Size
LipL 32F	**5'- CGCGGTCGACGCTTTCGGTGGTCTGCCAAGC-3'**	
LipL 32R	**5'-CGCGCTGCAGTTACTTAGTCGCGTCAGAAG – 3'**	**767bp**

Table 2. Infecting serovars of *Leptospira* identified using Microscopic agglutination test

Serovars	**No. of positive samples**	**Per cent positivity**
Australis	8	29.62
Autumnalis	5	18.51
Canicola	1	3.7
Pomona	1	3.7
Icterohaemorrhagiae	4	14.81
Grippotyphosa	2	7.40
Bataviae	3	11.11
Javanica	1	3.7
Pyrogenes	2	7.40
Total	27	

vaccinated animals. Adeyisun *et al.* (2006) pointed out the importance of incorporating the geographically prevalent serovars in vaccines.

Serological methods are most used for diagnosis of leptospirosis, but isolation allows definitive diagnosis of individual infections (Frietas *et al.,* 2004). Out of the 100 samples collected in the present study, eight isolates showed presence of distinct subsurface (Dinger's disc) growth in semi-solid EMJH media within three weeks to two months of incubation (Fig.1) and characteristic corkscrew motility upon DFM examination (Soman, 2004). The isolation of leptospires from clinical samples were found to be labour intensive and time consuming, requiring great skill and expertise (Thiermann, 1984). Overgrowth by contaminating organisms is another important factor interfering the successful isolation.

In recent years, PCR has proved to be an effective technique for rapid diagnosis of leptospirosis. Detection of leptospiral antigens directly from clinical samples enhances its practical utility, particularly in diagnosing acute infection (Ramdas *et al.,* 1997). Van eys *et al.* (1989) reported successful detection of leptospiral DNA from urine, while Merien *et al.* (1992) from serum samples of clinically suspected cases of leptospirosis. In the present study, eight canine isolates obtained by cultural isolation when subjected to PCR targeting *lipl32* gene. Positive amplicons of size of 767 bp was detected only in two samples (Fig.2). The remaining six isolates were found negative, which might be considered as non-pathogenic serovars (Levett *et al.*, 2005).

Based on the above results, the present study recommends the use of MAT for identification of *Leptospira* serovars from serum, while PCR can provide a rapid diagnostic method, particularly in acute infections.

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