REAL-TIME PCR BASED DETECTION OF LEPTOSPIRES IN BLOOD AND URINE OF DOGS

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THESIS

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DEPARTMENT OF VETERINARY MICROBIOLOGY
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DECLARATION

I hereby declare that this thesis, entitled **"Real-time PCR based detection of leptospires in blood and urine of dogs"** is a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Certified that this thesis, entitled **"Real-time PCR based detection of leptospires in blood and urine of dogs"** is a record of research work done independently by Subi T. K. (14-MVM-35) under my guidance and supervision and it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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We, the undersigned members of the advisory committee of **Subi T. K. (14-MVM-35),** a candidate for the degree of Master of Veterinary Science in Veterinary Clinical Medicine, Ethics and Jurisprudence, agree that this thesis entitled **"Real-time PCR based detection of leptospires in blood and urine of dogs"** may be submitted by **Subi. T. K. (14-MVM-35)** in partial fulfilment of the requirement for the degree.

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EXTERNAL EXAMINER

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SUBI T. K.

DEDICATED TO

MY PARENTS

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1. INTRODUCTION

Leptospirosis is an anthropozoonotic disease caused by *Spirochaetes* belonging to the genus *Leptospira*. It is a major threat in tropical and subtropical as well as in temperate countries affecting human, wild and domestic animals (Faine *et al*., 1999). Many of the south Indian states involving Kerala, are reporting a higher endemicity for this disease. A wide spectrum of hosts like cattle, horses, sheep, goats, pigs, dogs and carriers like rats pose a great threat to human. In human, the disease will lead to severe systemic illness with fatal complications. In domestic animals, the disease causes considerable economic loss as it is causing serious gynaecological problems, higher impact on production traits and finally death. In dogs, the disease is characterized by fever, vomiting, dehydration, collapse, hepatitis, nephritis and death (Faine, 1982).

Diagnosis of the disease in its acute stage is important to initiate appropriate treatment and prophylactic measures. The diagnosis of leptospirosis, especially in its early phases, is difficult, because of the varied clinical symptoms to make it often indistinguishable from other febrile illnesses (Levett, 2003). It is difficult to diagnose leptospirosis in an endemic region, using serological tests because of the presence of under - current antibodies, either due to prior infection or vaccination (Wolff and Bohlander, 1966). Thus, a rapid detection method are required for the implementation of appropriate medical measures and to conduct seroprevalence studies in the risk population.

Laboratory diagnosis of this disease is carried out by detection of the organisms in clinical specimens such as blood or urine, cultural isolation, molecular methods for the direct detection of leptospires or by serological test methods that helping the determination of antibodies (Levett, 2003). The demonstration of leptospires by dark field microscopy (DFM) in blood or urine of animals exhibiting the signs of acute leptospirosis is considered to be positive. However, approximately 10^4 leptospires need to present per mL of the clinical

sample for visualization of one cell per field by DFM and thus considered highly insensitive. Bacterial isolation from blood often fails because of the transient bacteraemia and often may not be associated with the characteristic clinical signs. Animal taken antibiotics before the collection of samples diminishes the chances of getting the bacteria in blood (OIE, 2008). Microscopic agglutination test (MAT) is the standard reference test for the serological identification of the organisms by virtue of its high sensitivity and specificity (Levett, 2001). However, the need of paired sera samples, expertise in performing and interpreting the results and handling of live cultures restrict the test to reference laboratories only. Further, MAT cannot distinguish the antibodies due to acute infection from that of past infection or due to vaccination (WHO, 2007). The molecular diagnostic techniques developed so far are mostly based on polymerase chain reaction (PCR) assays, which require well-established laboratory facilities and highly skilled personnel (Levett, 2003). Even though direct detection of leptospires from clinical samples were successfully accomplished by PCR, the interpretation of the results requires further analysis on agarose gel electrophoresis.

In order to circumvent the disadvantages of conventional PCR, real-time PCR was tested by many of the researchers. The procedure always in parallel with the PCR protocoal, but in this protocoal the DNA is concentration could be assessed during the progression of the reaction itself. Additionally, the real- time PCR enables the quantification of the target DNA, which would aid in understanding the gravity of infection, particularly in diseases like leptospirosis. A rapid, sensitive results could be achieved by means of this assay. Though the technique is costlier compared to the conventional PCR, it is considered as more advantageous, since it can detect the presence of multiple serovars involved in the disease, by performing real-time PCR and also enables the quantification of the organism in the clinical sample. The present study envisages the standardization of real-time PCR for the detection of leptospiral DNA from canine blood and urine samples.

The objectives are:

1. To standardise a real-time PCR for the diagnosis of canine leptospirosis

2. To detect the minimum detection limits of DNA in both PCR and real-time PCR

3. Sensitivity and specificity of both conventional and real-time PCR in comparison with Microscopic Agglutination Test

2. REVIEW OF LITERATURE

Leptospirosis is considered to be one of the zoonotic diseases geographically distributed mainly in the tropics, subtropics, and temperate climatic regions of the world caused by the pathogenic organism belonging to the genus *Leptospira*.

2.1 HISTORY

Leptospirosis is the general term that denotes all infections of man and animals caused by *Spirochaetes* of the genus *Leptospira*. The disease characterized by icterus and renal failure was first reported by Weil (1886).

Goldschmidt (1887) first used the term Weil's disease.

Stimson (1907) named as '*Spirochaeta interrogans'* based on morphology of the organism. Later on, *Spirochaeta interrogans* was renamed as *Leptospira interrogans (L. interrogans).*

Inada *et al*. (1916) observed *Leptospira* from guinea pig liver. It was inoculated with blood of patients suffering with Weil's disease.

"Ido *et al.* (1917) isolated *Leptospira* from a patient showing the symptoms and named as *Leptospira icterohaemorrhagiae (L. icterohaemorrhagiae*)".

Noguchi (1917) suggested a generic name "*Leptospira*" (fine coil).

2.2 CLASSIFICATION

The genus *Leptospira* belongs to the family *Leptospiraceae,* belonging to the order *Spirochaetales*. Genus *Leptospira* was divided into two species *i*.e., *L. interrogans* comprising the pathogens and *L. biflexa* comprising the saprophytes.

On the basis of serological reactions different serovars were identified and the serovars with common antigens were assembled into a serogroup (Quinn *et al.,* 2002).

According to WHO (2003), “two strains were considered to belong to different serovars if, after cross-absorption with adequate amount of heterologous antigen, more than 10 per cent of the homologous titre regularly remains in at least one of the two antisera in repeated tests”.

The committee on taxonomy of *Leptospira* of the international union of microbiological societies, in a meeting during International Leptospirosis Society Conference in the year 2002, approved that the genus and the species names will be italicized with the serovar names not to be italicized and written in the upper case first letter, e.g. *Leptospira interrogans* serovar Icterohaemorrhagiae (Srivastava, 2004).

Leptospira interrogans species comprised of 25 serogroups with more than 250 serovars (WHO, 2007).

Genetically, the genus *Leptospira* had been classified into the following 17 species *viz., L. interrogans, L. borgpetersenii, L. santarosai, L. inadi, L. noguchii, L. weilli, L. kirschneri, l. biflexa, L. meyeri, L. wolbachii, L. illini* and five other unnamed species *viz., Leptospira* genome species 1, *L.* genome species 2, *L.* genome species 3, *L.* genomespecies 4 and *L.* genomespecies 5 (Levett, 2001).

2.3 MORPHOLOGY

The genus *Leptospira* is characterised by fine coiling of the primary spirals. The name is derived from the Greek word *lepto* meaning thin or fine and Latin *spira* (coiled).

Leptospires are helicoidal organisms having a diameter of 0.1 µm and length of 6 to 20µm. One or both ends are usually hooked. Because of these characteristics it can pass through 0.1 -0.45 µm pore size membrane filters (Faine 1982).

The organisms are clearly visible by dark-field and much less clearly by phase contrast microscopy "The appearance and the motility of leptospires is determined by the nature of the growth medium. Three types of movements shown by leptospires are., rotation around the central axis, progressive movement and circular motion (Bharti *et al*., 2003)".

The diaminopimelic acid content of *Leptospira* serves to differentiate these organisms from treponemes and members of the genus coming under *Spirochaetes* (Cullen *et al*., 2004).

2.4 CULTURAL CONDITIONS

Dinger (1932) reported that in semisolid agar growth was found to be below the surface and known as Dinger's disc or ring (Dinger, 1932).

Leptospires could be isolated in enriched media containing vitamins B_1 and B12, long chain fatty acids and ammonium salts. Long chain fatty acids act as the sole source of carbon. Since the leptospires are having inherent toxicity of the free fatty acids, it should be added in bound form with albumin (Johnson and Gary, 1962).

The media contamination could be controlled by addition of a variety of selective agents like 5-Fluorouracil (5-FU), nalidixic acid, fosfomycin, or a mixture of rifamycin, polymyxin, neomycin, bacitracin, and actidione which used in the selective media for the initial isolation of leptospires (Johnson and Rogers, 1964).

The most widely used media are Ellinghausen, Mc Cullough, Johnson and Harris (EMJH) medium named for the individuals who first introduced it., they are Ellinghausen and McCullough, 1965; Johnson and Harris, 1967 or Tween 80/BSA medium containing oleic acid, bovine serum albumin (BSA) and Tween 80 (polysorbte). Sometimes a combination of Tween 80, Tween 40 and BSA is used.

Several liquid media were successfully used previously containing rabbit serum, bovine albumin or protein free media which helped in enrichment (Turner, 1970).

Leptospires grow well at pH 7.2 to 7.4 in rabbit serum or Tween 80 albumin media, while fastidious strains required the addition of either pyruvate (Johnson *et al*., 1973) or rabbit serum (Ellis *et al.*, 1976) for primary isolation.

Faine (1982) reported that the generation time of pathogenic *Leptospira* cultivated in laboratory media is relatively longer (about7-12 hrs). This may result in contamination of the culture with other organisms which may lead to the death of the leptospires.

Selective media containing antibiotics like polymyxin B, rifampicin and vancomycin were recommended to reduce contamination on primary isolation or to purify contaminated cultures (Myers and Varela-Diaz, 1973; Adler *et al*., 1986).

Leptopira are aerobic, with respiratory metabolism, which utilizes oxygen as the final electron acceptor and organisms required optimum temperature of 28^{0} C to 30^{0} C (Levett, 2001).

Cultures stored in sealed tubes in the dark at room temperatures below about 40^{0}C remained viable for months; Lyophilization (Annear, 1974) or freezing at -70^{0}C (Alexander *et al*., 1972; Palit *et al*., 1986) or in liquid nitrogen (Rossetti and

Auteri, 2008) are different techniques to achieve the organism to be stable for long term.

2.5 SEROPREVALENCE

2.5.1 Abroad

An Analysis of 464 serum samples of cows in Brazil revealed that the major cause of seroconversion was serovars Hardjo and Bratislava (Oliveira *et al*., 2001).

Alonso *et al*. (2001) in Spain found that *Leptospira interrogans* serovar Bratislava was found in 43 per cent of the herds and in eight per cent of the individual cattle.

Prescott *et al*. (2002) reported *Leptospira interrogans* serovar Autumnalis as the most prevalent serovar affecting dogs in Ontario.

In a seroprevalence study conducted for canine leptospirosis in Tehran between October 1998 and October 2000, out of 300 serum 93 (31.0 per cent) sera samples were found to be seropositive for leptospirosis (Rad *et al.,* 2004).

Adesiyun *et al*. (2006) reported the presence of *Leptospira* agglutinins in 61 out of 419 canine serum samples tested in Trinidad.

A prevalence study conducted for leptospirosis in canines in Taiwan (Lal *et al.,* 2006), could reveal a seropositivity of 23.0 per cent (72/313). The predominant serovar was Canicola (16 per cent), which was followed by Icterohaemorrhagiae (15.7 per cent), Pyrogenes (1.3 per cent), Bataviae (1 per cent) and Pomona (0.3 per cent).

Miller *et al*. (2007) reported that Australis and Zanoni were the most common serovars affecting dogs in North Queensland.

A seroprevalence study on canine leptospirosis was conducted in various parts of Australia (Zwijnenberg *et al.,* 2008). Among the 956 samples tested, 18 (1.9 per cent) had *Leptospira* agglutinins with *Leptospira interrogans* serovar Copenhageni as the predominant serovar.

In a seroprevalence study conducted in 52 dogs in Southwestern Nigeria, Okewole and Ayoola (2009) revealed that Grippotyphosa, Pomona and Bratislava were the most prevalent serovars associated with canine leptospirosis.

A serovar study was conducted in South Africa during the period 2008 to 2009, employing 530 samples of canines. Twenty were tested positive for seven different serovars of which, the serovars Canicola and Pyrogenes were found to be the most prevalent (Roach *et al.*, 2010).

2.5.2 India

The prevalence of leptospirosis in animals and humans has been reported based on sero-surveillance studies conducted in different parts of the country.

Srivastava *et al*. (1983) used MAT to detect antibodies to 16 serovars of *L. interrogans* and *L. biflexa* in serum samples throughout India. A total of 36 (10.1 per cent) of 355 cattle, 4 (5.9 per cent) of 68 buffalo, 12 (7.1 per cent) of 169 sheep, 5 (6 per cent) of 80 goats and 3 of 7 wild animals were found positive.

Autumnalis and Pomona were found to be the predominant serovars in a study conducted in Tamil Nadu employing sera of different domestic animals and humans (Ratnam *et al*., 1994).

In a seroprevalence study conducted during 1990-2000 by Srivastava and Kumar (2003), leptospiral agglutinins were detected in 15.8 per cent of 2601 cattle, followed by 9.9 per cent of 756 equine, 15.2 per cent of 551 sheep, 2.7 per cent of 443 buffaloe, 14.3 per cent of 271 goats and 19.1 per cent of 166 swine, in various states of the country.

Soman (2004), in a seroprevalence study conducted in Thrissur, Kerala, reported that Pomona and Australis as most prevalent serovars among canines.

Koteeswaran (2006) carried out a seroprevalence study in Tamil Nadu and reported a seropositivity of 57.47 per cent among domestic animals, 72.73 per cent in wild animals in captivity and 37.03 per cent in rodents, with an overall per cent positivity of 56.68. The predominant serovar among domestic animals was Australis (20.28 per cent), followed by the other serovars *viz.*, Hebdomadis (14.31 per cent), Sejroe (12.85 per cent), Pomona (12.51 per cent), Pyrogenes (8.23 per cent), Tarassovi (7.89 per cent), Autumnalis (6.42 per cent), Canicola (5.41 per cent), Ballum (4.28 per cent), Icterohaemorrhagiae (3.72 per cent), Grippotyphosa (2.37 per cent) and Javanica (1.75 per cent).

Swapna *et al*. (2006) conducted a seroprevalence study in Kerala, among agricultural workers, hospital sanitary workers, veterinarians and labourers. The most common serovar identified in the high risk groups was Pomona (26.5 per cent), followed by Shermani (19.5 per cent), Canicola (16 per cent), Bataviae (13.5 per cent), Autumnalis (11 per cent), Djasiman (10.5 per cent), Tarassovi (10 per cent), Icterohaemorrhagiae (7 per cent), Australis (6.5 per cent), Hebdomadis (4.5 per cent), Hardjo (3 per cent), Ballum (2.5 per cent), Cynopteri (2.5 per cent), Sarmin (2.5 per cent) and Patoc (2.5 per cent).

A seroprevalence study was conducted among the cattle and buffaloes by Mariya *et al*. (2006) in Uttaranchal, Tamil Nadu and Uttar Pradesh. A total of 68 sera samples came positive among the 321 samples tested and serovar Sejroe was found to be predominant (6.2 per cent).

Balakrishnan *et al*. (2008) reported *L. interrogans* serovar Australis as an emerging serovar in Tamil Nadu.

Sumathi *et al.* (2008) revealed that leptospirosis occurred throughout the year, although the number might increase during the monsoon season (June to January). This emphasized the importance of a polluted environment which is an important epidemiological risk factor.

Sariprabha (2010) conducted a seroprevalence study of canine leptospirosis in Thrissur, Kerala and reported that the serovar Australis was the most prevalent one (38.33 per cent), followed by Grippotyphosa (18.33 per cent), Pomona (18.33 per cent), Canicola (15.0 per cent), Icterohaemorrhagiae (13.33 per cent), Javanica (10.0 per cent), Patoc (8.33 per cent), Autumnalis (6.67 per cent) and Pyrogenes (6.67 per cent).

Abhinay (2011) in a seroprevalence study conducted in Thrissur, Kerala, reported that Autumnalis and Pomona as most prevalent serovars among canines.

Vamshi (2012) in a seroprevalence study conducted in Thrissur, Kerala, reported that Autumnalis and Pomona as most prevalent serovars among canines.

Visakh (2015) in a seroprevalence study conducted in Thrissur, Kerala, reported that Australis and Grippotyphosa as most prevalent serovars among canines.

2.6 ANTIGENS OF LEPTOSPIRES

Research has involved identification of antigens of leptospires which could be of importance for diagnostic and prophylactic use. So far, lipopolysaccharides (LPS), surface proteins and outer membrane proteins of several leptospiral serovars have been identified as putative candidates (Srivastava and Tiwari, 2000).

2.6.1 Whole cell antigens

Whole cell antigens were tried in various serological assays like, macroscopic agglutination (Mailloux *et al.,* 1974), microcapsule agglutination (Arimitsu *et al*., 1982) and ELISA (Terpstra *et al*., 1985; Yersin *et al*., 1999).

The disaccharide epitope in the immunodominant moiety of whole cell preparations was present in non pathogenic leptospires as well as a diverse group of non leptospiral species that resulted in cross-reactivity (Matsuo *et al*., 2000). As a result, these tests suffered from low sensitivity and identified less than 50 per cent of patients presenting the early phase of leptospirosis (Guerreiro *et al.,* 2001).

2.7 DIAGNOSIS OF LEPTOSPIROSIS

Bacteriological diagnosis of leptospirosis is achieved by methods like isolation and DFM. Confirmatory diagnosis can be provided by isolation of the organism, but it was reported to be time consuming and cumbersome (Levett and Whittington, 1998).

Molecular diagnostic techniques like PCR required well-established laboratory facilities and highly skilled personnel (Levett, 2003).

It is opined that Dark field microscopy required the presence of a minimum of 10^4 leptospires per milli litre, to observe one cell per field, making it less sensitive (Ahmad *et al.,* 2005).

2.7.1 Serological diagnosis

Many serological screening tests for leptospiral antibodies have been developed in earlier times which include complement fixation test (Pot and Dornickx, 1936), microcapsule agglutination test (Arimitsu *et al*., 1982) and indirect haemagglutination test (Levett and Whittington, 1998). All these tests

were proved to be less sensitive than the gold standard test, MAT (Guerreiro *et al*., 2001).

2.7.1.1 Microscopic agglutination test

The antigenic variability of leptospiral lipopolysaccharide (LPS) was considered to be one of the limiting factors for serodiagnosis of the disease. The MAT has been the "gold standard" confirmatory test for the past several years and is most likely based on the seroreactivity with LPS antigens.

Microscopic agglutination test is said to be a complex test, difficult to perform, interpret and reported to require the maintenance of live cultures also (Turner, 1968).

Being serovar specific, MAT may not be of true diagnostic value in diagnosing the disease (Champagne *et al.,* 1991).

The microscopic agglutination test was reported to detect both IgG and IgM antibodies (Chernukha *et al.,* 1976 and Romero *et al.,* 1998).

The repeated weekly subculture of large numbers of strains presents hazards for laboratory workers (Pike, 1976). Formalinized antigens were used in the MAT to overcome some of the difficulties associated with the use of live antigens. Titers obtained with these antigens were lower and more cross-reactions could be detected (Sulzer and Jones, 1978 and Faine, 1982).

Paired sera from the suspected individuals are required to confirm a diagnosis with certainty. Moreover, patients with fulminant leptospirosis died before seroconversion (Ribeiro *et al*., 1994).

Serological observations made by MAT were reported to be insensitive in early acute-phase specimens (Cumberland *et al*., 1999). The need to assess agglutination by dark field microscopy and to maintain a large battery of live

leptospiral antigens in culture restricts the use of the MAT to a few reference laboratories worldwide (Guerreiro *et al*., 2001).

An epidemiological study conducted by Ooteman *et al.* (2006) in Brazil, MAT was used for confirmatory diagnosis, confirmation was done by observing a four-fold increase in titer between paired serum samples or a titer greater than 800 in a single serum sample.

2.7.2 Molecular techniques

2.7.2.1 Polymerase Chain Reaction (PCR)

The PCR technique was first attempted for the detection of leptospires in the urine samples of bovine and reported it as a useful technique in the detection of leptospires (Van Eys *et al.*,1989)

In a comparative study, PCR was found to be more sensitive than either MAT or culture methods for the diagnosis of human leptospirosis (Merien *et al*., 1992).

The primers described by Merien *et al.* (1992) amplified a 332 bp fragment of both pathogenic and nonpathogenic leptospires. They also reported that PCR could be an ungauged tool for early diagnosis of the disease, especially when the clinical expression of the disease is confusing.

Gravekamp *et al.* (1993) introduced G1/ G2 primers which enabled the amplification of nucleic acid specific for pathogenic leptospires. But, these primers did not amplify several pathogenic serovars like *L. kirschneri* which resulted in false negative results.

According to Brown *et al*. (1995), both PCR and culture were more often positive for sera than for urine samples and according to them, PCR could detect leptospires in sera even before the development of antibodies.

Senthilkumar *et al.* (2001) reported the efficacy of PCR in detecting leptospires from blood, urine and milk in comparison with DFM.

Molecular diagnostic techniques like PCR required well-established laboratory facilities and highly skilled personnel (Levett, 2003).

Ooteman *et al.* (2006) observed a higher number of positive cases verified by PCR (13 to 19 per cent) and IgM ELISA (3 to 7 per cent) among the 45 unconfirmed cases by MAT, demonstrated the value of PCR in the early diagnosis of human leptospirosis.

According to Vijayachari *et al.* (2006), the early diagnosis of leptospirosis could be achieved by the amplification of leptospiral genomic DNA in clinical samples using PCR. Polymerase chain reaction on serum samples using two sets of primers (G1/G2 and B64I/B64II) showed 95.2 per cent sensitivity and 91.4 per cent specificity, when compared with the isolation and or paired MAT.

Cheema *et al.* (2007) developed a PCR based on *lipl21* and *lipl32* genes for the detection of pathogenic leptospires in animals.

2.7.2.2 Real-time PCR assay

Haake *et al*. (2000) developed a real-time assay using SYBR Green chemistry, in which the major outer-membrane lipoprotein *lipl32* was the target gene.

According to Schmittgen *et al*. (2000) real-time PCR more precise and displays a greater dynamic range than ordinary PCR. They observed that SYBR green and Taqman assays produced comparable sensitivity and dynamic range, while SYBR green detection was more precise compared to other and produced a more linear decay plot than Taqman assay.

A study conducted by Fearnley *et al.* (2008) reported that the confirmation of results in conventional PCR required the manual electrophoresis and visualization under the gel documentation, which may increase the time required for the result delivery and this hurdle could be easily overcome by employing the real time PCR technique.

Smythe *et al.* (2002) reported that on comparing with the culturing and conventional techniques, the real time PCR assay required only two cells for the result confirmation. It was said to be a rapid and effective tool without any further delay in the result delivery.

A study conducted by Smythe *et al.* (2002) reported that the advantage of SYBR green lays in its cost effectiveness when compared to FRET and Taq man probes, but the main disadvantage with the SYBR green chemistry is its non specificity because of the chance of binding with any double stranded DNA within the sample which resulted in false positive signals.

Palaniyappan *et al*. (2005) reported that culturing of tissues from the experimentally infected hamsters could detect the organism on second day, whereas both conventional as well as real time PCR could detect the presence of the organisms on the day of infection itself.

In order to mask the draw backs of conventional PCR assays, a real time PCR assay was developed targeting the highly immunogenic *lipl32* gene, which is classified under Haemolysis associated protein or Hap-1 (Levett *et al.*, 2005) seen only in the pathogenic leptospires.

Merien *et al.* (2005) reported that real-time assay could be used as a fast as well as effective method to calculate the number or quantity of the *Leptospira* in human infections and it had numerous advantages when comparing with the conventional techniques.

"Levett *et al.* (2005) developed a real- time quantitative PCR based on SYBR Green fluorescence for the detection of pathogenic leptospires by using a 423 bp target on the *lipl32* gene. Reactions were monitored by SYBR green fluorescence and melt curve analysis. Representative serovars from 16 species of *Leptospira* and over 40 species of other bacteria and fungi were tested. Positive results were obtained with all pathogenic leptospiral serovars, with the exception of *Leptospira fainei* serovar Hurstbridge. The analytical sensitivity of the assay was three genome equivalents per reaction; approximately 10 genome equivalents were detectable in human urine."

Merien *et al.* (2005) developed a cost effective real time PCR assay based on SYBR Green chemistry for the detection of pathogenic leptospires in human sera and observed a sensitivity of 50 leptospires/ mL of the sample.

Palaniappan *et al.* (2005) evaluated *lig* gene based real time PCR for the detection of pathogenic leptospires. They concluded real time assay as a rapid and sensitive diagnostic method as compared to culture and conventional PCR.

Ahmad *et al*. (2012) performed the testing of blood and urine samples from canines having leptospiraemia using a SYBR green based real time PCR and observed a sensitivity of 91.7 per cent and specificity of 90.0 per cent in comparison with culturing.

Karuniawati *et al.*, (2012) reported that a good temperature setting is required for an effective amplification of the target gene in PCR. They recorded that the higher temperature would affect the attachment of the primer with the template and lower temperature leads to nonspecific primer binding.

Karuniawati *et al.* (2012) optimized a real-time PCR to detect *Leptospira* spp. in human blood and urine specimens and observed that the minimum limit of detection for DNA was150 fg/µL and 1470 fg/µL from blood and urine, respectively.

Use of a QIA amp mini kit was a more convenient method to isolate the leptospiral DNA from the patient's samples such as sera and urine (Smythe *et al*., 2002 and Karuniawati *et al.,* 2012).

An experiment conducted by Agampodi *et al*. (2012) evaluated the sensitivity and specificity of a quantitative PCR targeting the 16S ribosomal RNA gene from whole blood and serum and observed to have the respective values of 18.4 per cent and 51 per cent, respectively.

Picardeau *et al.* (2014) reported that the amplification of real-time PCR will be completed in less than one hour and was less sensitive to contamination as the conventional PCR requires post thermal cycling procedures such as visualization under agarose gel electrophoresis which may lead to contaminating the tubes.

3. MATERIALS AND METHODS

All the reagents used in the study were of molecular biology grade, obtained from Sigma-Aldrich, Merck Genei, Immunology Consultants Laboratory (ICL), USA, MBI Fermentas and Sisco Research Laboratories (SRL) private limited. The source for these reagents has been provided in the appropriate places in the text. Readymade medium from Difco Laboratories was used for culturing *Leptospira*. Glassware of Borosil brand and plastic ware of Tarsons brand were used in the study.

3.1 REFERENCE STRAINS OF *Leptospira*

The following serovars of *Leptospira*, procured from the National *Leptospira* Reference centre, Regional Medical Research Centre, Port Blair, Andaman and Nicobar Islands and maintained in the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy, were used in the study. The main serovars included *Leptospira interrogans* serovar Australis, Autumnalis, Canicola, Pomona, Bataviae, Hebdomadis, Icterohaemorrhagiae, Pyrogenes and Grippotyphosa.

3.2 MEDIA

3.2.1 Leptospira culture medium base (Difco)

Leptospira culture medium base with supplement was employed for growth and maintenance of the leptospires.

3.2.2 Supplement (Albumin supplement)

For the preparation of supplement, the following stock solutions were made in distilled water (expressed in grams per 100 mL)

Zinc Sulphate. 7 H_2O	0.1
Calcium Chloride. 2 H_2O	1.0
Magnesium Chloride. 6 H_2O	1.0
Ferrous Sulphate. 7 H_2O	0.5
Copper Sulphate. 5 H_2O	0.3
Tween 80	10
Vitamin B_{12}	0.02

The supplement was prepared by adding 20 g of bovine serum albumin (BSA) fraction V (SRL) in 100 mL of distilled water. The following quantities of stock solutions were added slowly to the albumin solution, while it was being stirred.

Zinc Sulphate. 7 H_2O	2 mL
Calcium Chloride. 2 H_2O	2 mL
Magnesium Chloride. 6 H_2O	2 mL
Ferrous Sulphate. 7 H_2O	20 mL
Copper Sulphate. 5 H_2O	0.2 mL
Tween 80	25 mL
Vitamin B_{12}	2 mL

The pH of the BSA solution was adjusted to 7.4 using 1N sodium hydroxide and the final volume was made up to 200 mL with distilled water and then sterilized by filtration using syringe filter (0.22 µM).

3.2.3 Preparation of 5-Fluorouracil solution (5-FU)

To avoid contamination, the medium was supplemented with 5-Fluorouracil (5- FU) (HiMedia) which was prepared as follows. One hundred milligram of the 5-FU was added to five millilitre of sterile triple distilled water. To this, 0.2 mL of 0.1N sodium hydroxide was added and the volume was made up to 10 mL with sterile triple distilled water. The solution was sterilized by filtration through a 0.22 µm membrane filter and stored at -20^{o}C. One millilitre of this solution was added to 100 mL of liquid or semi-solid medium to obtain a final concentration of 100 µg/mL of medium.

3.2.4 Leptospira liquid culture medium

The *Leptospira* liquid culture medium was prepared by dissolving 0.23 g *Leptospira* liquid medium base (Difco) in 90 mL of triple distilled water. The medium was autoclaved at 121^{o}C for 15 min. at 15 lbs pressure and allowed to cool to 40-50^{o}C. Supplement was added at 10 per cent level to the base medium. Further, one millilitre of 5-FU solution was added to 100 mL of liquid or semi-solid medium so as to obtain a final 5-FU concentration of 100 µg/mL in the medium. Medium was dispensed in three to five millilitre quantities in screw capped tubes. The tubes were checked for sterility by incubating at 37^{o}C for 48 h and then stored at 4^{o}C.

3.2.5 Leptospira semi-solid culture medium

Leptospira semi-solid medium was prepared by adding 0.5 per cent bacteriological agar to *Leptospira* liquid medium. The medium was autoclaved at 121^{o}C for 15 min at 15 lbs pressure and was allowed to cool. Supplement and 5-FU were added as described above (3.1.2.4). The medium was distributed in aliquots of three to five millilitre in screw capped tubes. Sterility of the medium was tested by incubating the medium for 48 h at 37 ^{o}C and then stored at 4^{o}C.

3.3 SEROLOGICAL METHODS

3.3.1 Microscopic agglutination test

The MAT was carried out using live *Leptospira* organisms following OIE (2008). Five to seven days-old liquid culture of live leptospires with a density of approximately 2 X 10^8 leptospires per millilitre was used as antigen. The test was carried out in a 96 well 'U' bottom microtitre plate.

Serum dilutions were made in 96 well microtitre plates for which 798 µL of PBS was mixed with 2µL (1:400) of serum. From the1:400 diluted serum sample, 50 µL was added to each of the 8 wells in each column of the microtitre plate which serve as the test. In the second row 50µL of PBS was added which served as antigen control. Different serovars (50 µL) were added respectively to each rows. The plates were closed with lid and incubated at 37 ^{o}C for two to four hours. A drop (10 µL) of the mixture was placed on clean grease-free slide and the wet preparation was examined without coverslip using 10X objective of the dark field microscope (Carl Zeiss) for the presence of agglutination.

3.3.2 Polymerase chain reaction (PCR)

3.3.2.1 Primers

The sequences of the primers were as follows.

Table.1

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

3.3.2.2 Reagents for test proper

3.3.2.2a PCR reaction buffer (10X)

500 mM KCl, 100 mM Tris-HCl pH 9.0 and 15 mM $MgCl_2$ (Bangalore, GeNei).

3.3.2.2b Deoxyribonucleotide triphosphate (dNTP)

dNTP mix 10 mM (dGTP, dCTP, dATP, dTTP in equal volume) was procured from Bangalore, Genei.

3.3.2.2c Taq DNA polymerase

Taq DNA polymerase enzyme with a concentration of 3U/µL was procured from Bangalore, Genei.

3.3.2.3 Reagents for agarose gel electrophoresis

3.3.2.3a Trisborate EDTA buffer (TBE) (Stock solution 5X) pH 8.0

Tris base	54 g
Boric acid	27.5 g
EDTA (0.5 M, pH 8.0)	20 mL
Distilled water to	1000 mL

Dissolved in 900 mL distilled water and pH adjusted to 8.0. The volume was made up to 1000 mL, was then filtered and stored at 4º C. The stock solution was diluted to 1X before use.

3.3.2.3b Agarose gel (1.5 per cent)

Agarose low EEO (Bangalore Genei, Bangalore)	1.5 g
1X TBE buffer	100 mL

3.3.2.3c Ethidium bromide

Ethidium bromide	1 g
Distilled water	100 mL

Stored at 4ºC in amber coloured bottle.

3.3.2.3d Gel loading buffer

Bromophenol blue	0.25 g
Sucrose	40 g
Distilled water	100 mL

Stored at 4ºC.

3.3.2.3e DNA molecular size (Fermentas, USA)

100 DNA ladder. Stored at 4º C.

3.3.2.4 DNA extraction

Deoxy-ribonucleic-acid was extracted from 200µL of EDTA anti coagulated whole blood samples and reference strains using QIA-AMP blood DNA extraction kit (QIAGEN, Hildan, Germany). The DNA extraction was carried out according to the manufacturer's instruction. The DNA was quantified using nanodrop DNA quantifier (Thermo electron scien. Instru. USA nanodrop 2000C model) and stored at -20ºC until further processed.

3.3.2.5 Reconstitution and Dilution of Primers

The primers obtained in lyophilized form were reconstituted in sterile triple distilled water to a concentration of 200 pM/µL and the tubes were spun briefly. The stock solution was distributed into 10 µL aliquots and stored at -20ºC until use. The working solution was made by diluting the stock solution 10 fold to obtain a concentration of 20 pM/µL before using for PCR.

3.3.2.6 Setting up of PCR (Test proper) for amplification of lipl32 gene

For the amplification of *lipl32* gene, the reaction was carried out in a 25 µL reaction tube. The master mix was prepared by combining the following reagents in 20 µL volume- 14 µL nuclease free water, 2.5 µL 10 X PCR buffer, 1 µL 10mM dNTP mix, 1 µL (20pmol) of each of the forward and reverse primers and 0.33 µL of *Taq* DNA polymerase (3 U/µL). To each PCR tube, 20µL of master mix and 5 µL of template DNA were added.

One negative control without template DNA was included to monitor any contamination. The tubes were spun briefly and placed in PCR thermal cycler (Eppendorf Master cycler) for amplification. The amplification cycle for *lipl32* gene was given in Table 2.

Table 2. Amplification cycle for *lipl32* gene

Initial denaturation	94° C for 4minutes	35cycles
Denaturation	94° C for 1 minutes	
Annealing	60° C for 45seconds	
Extension	72° C for 2 minutes	
Final extension	72° C for 10 minutes	

3.3.2.7 Detection of PCR Products

3.3.2.7a Submarine Agarose Gel Electrophoresis

The authenticity of PCR product was confirmed by its size in one per cent agarose gel (3.1.3.2e). The PCR products were detected by electrophoresis in a one per cent agarose gel in 1X TBE buffer (3.1.3.2d). Agarose was dissolved in TBE buffer (1X) by heating and cooled to 50ºC. To this, ethidium bromide (3.1.3.3.c) was added to a final concentration of 0.5µg/mL. The comb was kept in proper position before pouring agarose. Once the gel was set, the comb was removed gently and placed the gel tray in the buffer tank. Subsequently, TBE buffer (1X) was poured to cover the gel completely. The PCR product (5 µL) was mixed with one microlitre of 6X gel loading buffer **(3.1.3.3.d)** and the samples were loaded into the respective slots carefully. A 100bp DNA ladder (0.5 µg/µL) (Fermentas) was used as molecular size marker. Electrophoresis was carried out at

50 volts for 45 minutes or until the bromophenol blue migrated more than two-third of the length of the gel.

3.3.2.7b Recording of the results

The gel was visualized under UV trans-illuminator and the results were documented in a gel documentation system (BIORAD, USA)

3.3.3 Real-time PCR

3.3.3.1 Standardization of Real-time Polymerase Chain Reaction

3.3.3.1a. Primer designing and Real-time PCR standardization

Primers for real-time PCR of *lipl32* genes were taken from the standard article (Levett *et al.,* 2005). Primers were custom synthesised commercially (Sigma-Aldrich, Bangalore) and obtained in lyophilised form. The primer sequences used for q-PCR for *lipl32* gene are listed below:

*lipl*32 F1 - 5’CATTCATGTTTCGAATCATTTCAAA3’
*lipl*32R1 - 5'GGCCCAAGTTCCTTCTAAAAG3'

3.3.3.1b Primer dilution

The lyophilised primers were spun in order to prevent loss of contents. A stock solution of 100 pM/µL was prepared by addition of nuclear free water according to the manufacturer’s specification. It was kept in room temperature for one hour and then working solution of 20 pM was prepared in a fresh sterile 1.5 mL eppendorf tube, labelled and stored in -20ºC.

3.3.3.1c Setting up of Real-time PCR (Test proper)

The PCR was carried out in volume of 12.5 µL in 0.2 mL PCR tubes. The PCR was standardized for different gradients of temperatures using Maxima SYBR Green qPCR Master Mix (2X) with ROX (Thermo Scientific). Thermo Scientific Maxima SYBR Green /ROX q-PCR Mastermix (2X) is a ready to use

solution optimized for quantitative real time PCR. The master mix includes Maxima Hot Start *Taq* DNA polymerase and dNTPs in an optimized PCR buffer. It contains SYBR Green I dye supplemented with ROX passive reference dye. Duplicates of technical replicates were used. In addition, a non-template control (NTC) for the gene to check for primer-dimer, a positive control made with DNA extracted from leptospiral culture, a negative control (nuclease free water) were included in every plate. Reactions were carried out in Applied biosystem step I plus thermal cycler using 8-well plates, sealed by an adhesive seals after pipetting all reagents into the wells. Reaction mixture and thermal cycling parameters are given in Tables

Table 3. Reaction mix used for real-time PCR

Sl. No.	Component	Volume(µL)
1	Maxima SYBR Green qPCR Master Mix	6.25
2	Forward primer (20 pM/µl)	1
3	Reverse primer (20 pM/µl)	1
4	Template DNA	1
5	Nuclease free water	3.25
6	Total	12.5 µL

Table 4. Thermal cycling parameters used for qPCR

Step	Temperature, ^{0}C	Time	Number of cycles
Initial denaturation	94	4min	1
Denaturation	94	30sec	40
Annealing/Extension	60	30sec	40

3.3.3.1d Steps in lipl32 amplification using Real-Time PCR System

1. The Applied biosystem step I plus connected with computer system was switched on. The thermal profile and page layout was set up according to the samples added to the plate.

2. All the reagents were thawed and master mix was added to 0.2 mL PCR tube with total volume of 12.5 µL in each tube. Template (1 µL) was added separately in each tube and the mixture was centrifuged. The plate was placed on the dock, 12.5 µL of above mixture was added in each well.

3. After adding, the plate was sealed properly using the adhesive seal with the help of a squeegee.

4. The plate was centrifuged for 2500 rpm for three minutes.

5. The plate was carefully placed in the Step I plus Real-Time PCR system block aligning with the notch and the lid was closed.

6. Start run button in the computer screen was clicked and the run was monitored.

The PCR products were confirmed by gel electrophoresis using ethidium bromide stained two per cent agarose gel and documented in gel documentation system (Bio-Rad, USA).

3.3.3.2 Determination of minimum detection limit of DNA for PCR and Real time PCR

The detection limit for PCR and real-time PCR were determined using DNA extracted from standard strain of *Leptospira* serovar Australis culture. The concentration was measured by using Thermo-scientific nano-drop (spectrophotometer). Real-time PCR was carried out by using *Leptospira* culture which was serially diluted to tenfold. The amplification plot showed cycle number on the X axis and Delta Rn (Fluorescence) on the Y axis which determined the minimum detection limit of DNA, which was assessed by measuring the concentration of the sample preceding sample for which there was no

amplification. The specific amplicons with desired size showed a single peak by Melt curve analysis.

In conventional PCR, the detection limit was detected by serial dilution of the extracted DNA and all dilutions were tested as templates in PCR. Further confirmation was also carried out by agarose gel electrophoresis.

3.4 STATISTICAL ANALYSIS OF RESULTS

3.4.1 Comparison of MAT and PCR

The relative sensitivity, specificity and accuracy of the PCR for serodiagnosis of canine leptospirosis were evaluated in comparison to the MAT Sensitivity = a/(a+c) x 100, where, ‘a’ is the number of sera positive by PCR and MAT, ‘c’ the number of sera positive by MAT but negative by PCR.

Specificity = d/ (b+d) x 100, where’d’ is the number of sera negative by PCR and MAT, ‘b’ the number of sera negative by MAT but positive by PCR.

Accuracy = a+d/ a+b+c+d x 100

3.4.2 Comparison of MAT and Real-time PCR

The relative sensitivity, specificity and accuracy of the real-time PCR for serodiagnosis of canine leptospirosis were evaluated in comparison to the MAT Sensitivity = a/(a+c) x 100, where, ‘a’ is the number of sera positive by real-time and MAT, ‘c’ the number of sera positive by MAT but negative by real time.

Specificity = d/ (b+d) x 100, where’d’ is the number of sera negative by real time and MAT, ‘b’ the number of sera negative by MAT but positive by real time.

Accuracy = a+d/ a+b+c+d x 100

Statistical test was done with Chi-square test using SPSS software (version.20). Comparison among MAT, real time and PCR was found out using this test.

4. RESULTS

4.1 MAINTENANCE OF LEPTOSPIRAL CULTURES

The *Leptospira* serovars maintained in the Department of Veterinary Microbiology were used in the present study. For maintenance, EMJH medium (Difco) with albumin supplement was employed and sub cultured at seven day intervals and incubation was carried out at 28-30^{0}C. In addition, the stock cultures were maintained in semi-solid medium with sub culturing at one-month interval.

4.2 IDENTIFICATION OF LEPTOSPIRES

Under dark field microscope, the live leptospiral organisms were found tightly coiled and actively motile. The motility observed was of both spinning and bending. In highly concentrated cultures, the organisms formed entangled masses. No contaminants were observed in most of the time when streaked on blood agar plates for purity checking of the cultures. If there was contamination, the cultures were purified by filtration.

4.3 ISOLATION OF DNA

Deoxyribonucleic acid was isolated from plasma and urine samples obtained from dogs suspected for canine leptospirosis. The concentration and purity was checked in nano-drop spectrophotometer.

4.4 DIAGNOSIS

4.4.1 Microscopic Agglutination Test

A total of 75 canine serum samples from leptospirosis suspected dogs were tested using MAT, among which 36 (48 per cent) were found to have titre of ≥ 1:400 and hence concluded positive for leptospirosis. The infecting serovars identified with MAT are depicted in table 6.

4.4.2 Polymerase chain reaction

The outer membrane protein, *lipl32* gene conserved for pathogenic serovars was used for amplification. The reaction was carried out in a 25 µL reaction tube. The master mix was prepared by combining the following reagents in 20 µL volume- 14 µL nuclease free water, 2.5 µL 10 X PCR buffer, 1 µL 10mM dNTP mix, 1 µL (20pmol) of each of the forward and reverse primers and 0.33 µL of *Taq* DNA polymerase (3 U/µL). Annealing temperature optimized as 60^0 C for 45 seconds

Agarose gel electrophoresis of the amplified product using *lipl32* primers revealed an amplicon of 767 bp corresponding to the genus *Leptospira* when viewed under gel documentation system. In the negative control, no amplicon could be noticed (Fig. 6).

4.4.3 Real-time PCR

Real-time PCR was standardized using *lipl32* primers. The PCR was carried out in volume of 25 µL in 0.2 mL PCR tubes. The PCR was standardized for different gradients of temperatures using Maxima SYBR Green qPCR Master Mix (2X). Duplicates of technical replicates were used. Polymerase chain reaction was standardized for different gradients of temperatures. The DNA extracted from cultures kept as positive control and negative control was made up with nuclease free water. The master mix was prepared by combining the following reagents in 12.5 µL volume- 3.25µL nuclease free water, 6.25 µL Maxima SYBR Green qPCR Master Mix (2X), 1 µL (20pmol) of each of the forward and reverse primers. Annealing temperature optimized as 60^0C for 30 seconds gave an optimal result. Amplification plot (Fig.1) and melt curve was generated. Melt curve revealed a single peak for the gene which indicated the absence of nonspecific products (Fig.2). Then, the amplicons were visualized in three per cent agarose gel (Fig.5) which showed a 323 bp size.

4.4.3.1 Determination of cut off values

The concentration of DNA extracted from the leptospiral culture was 253 ng/ µL. Serial dilutions of the extracted DNA were tested as template in real-time PCR assay to find out the minimum detection limit. It was found that the real-time PCR could detect a minimum DNA concentration of 9 ng/ µL.

4.4.3.2 Test proper

The results of MAT, PCR and real-time PCR and are given in table 5. Among 75 samples, 39 were positive for real-time PCR, 36 were found to be positive with MAT and 30 were recorded as positive in PCR (Table 5).

4.4.4 Comparison of MAT and Conventional PCR

Among 75 canine sera examined, 36 samples showed positive MAT at 1:400 dilution and among this 30 samples could be detected positive using conventional PCR. The sensitivity, specificity and accuracy of PCR as relative to MAT was calculated to be 80.55 per cent, 97.4 per cent and 89 per cent, respectively (Table 7).

4.4.5 Comparison of MAT and Real -time PCR

The results of the real-time PCR were also compared with that of MAT. Among the 75 canine sera examined, 36 (48 per cent) exhibited positive agglutinins at 1:400 dilution for leptospirosis and 39 samples (52 per cent) demonstrated a positive amplification on real-time PCR assay. The sensitivity, specificity and accuracy of real-time PCR as compared to MAT was calculated to be 91.6 per cent, 84.61 per cent and 88 per cent, respectively (Table 8).

Table 5. Results of MAT, PCR and Real-time

Sl. No.	MAT	PCR	Real-time PCR
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	-	+	+
6	+	+	+
7	+	+	+
8	+	+	+
9	+	+	+
10	+	+	+
11	+	+	+
12	+	+	+
13	+	+	+
14	+	+	+
15	+	+	+
16	+	+	+
17	+	+	+
18	+	+	+
19	+	+	+
20	+	+	+
21	+	+	+
22	+	+	+
23	+	+	+
24	+	+	+
25	+	+	+
26	+	+	+

27	+	+	+
28	+	+	+
29	+	+	+
30	+	+	+
31	+	-	+
32	+	-	+
33	+	-	+
34	+	-	-
35	+	-	-
36	+	-	-
37	-	-	+
38	-	-	+
39	-	-	+
40	-	-	+
41	-	-	+
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65	-	-	-
66	-	-	-
67	-	-	-
68	-	-	-
69	-	-	-
70	-	-	-
71	-	-	-
72	-	-	-
73	-	-	-
74	-	-	-
75	-	-	-

Table 6. Infecting serovars identified using MAT

Leptospira interrogans Serovars	No. of positive samples	Per cent positivity
Australis	12	33.33
Autumnalis	7	19.44
Canicola	2	5.55
Pomona	3	8.33
Icterohaemorrhagiae	4	11.11
Grippotyphosa	4	11.11
Bataviae	1	2.77
Hebdomadis	3	8.33
Pyrogenes	0	0

Table 7. Comparison between MAT and PCR

	Microscopic agglutination test			
Polymerase Chain Reaction		Positive	Negative	Total
	Positive	29(a)	1(b)	30(a+b)
	Negative	7(c)	38(d)	45(c+d)
	Total	36(a+c)	39(b+d)	75(a+b+c+d)

Sensitivity = 80.55 per cent

Specificity = 97.40 per cent

Accuracy = 89 per cent

Table 8. Comparison between MAT and Real-time PCR

	Microscopic agglutination test			
Real-time PCR		Positive	Negative	Total
	Positive	33 (a)	6 (b)	39
	Negative	3 (c)	33 (d)	36
	Total	36 (a+c)	39 (b+d)	75(a+b+c+d)

Sensitivity = 91.60 per cent

Specificity = 84.61 per cent

Accuracy = 88 per cent

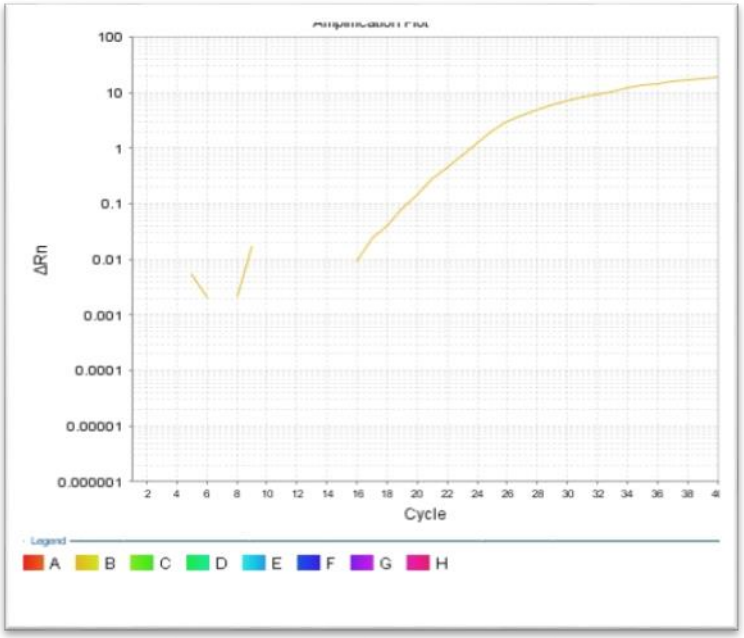

Fig. 1. Standardization of *lipl32* gene showing amplification from a cycle no. 16

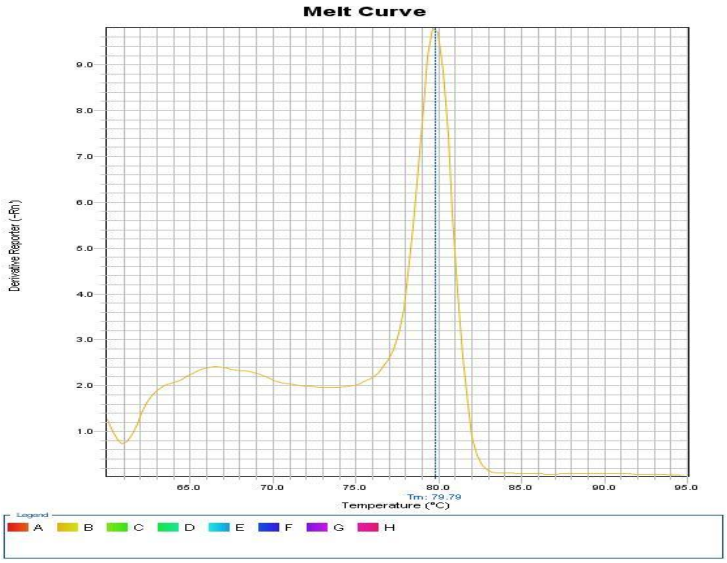

Fig. 2. Melt curve generated during the standardization of real-time PCR using *lipl32* (Tm value: 79.79)

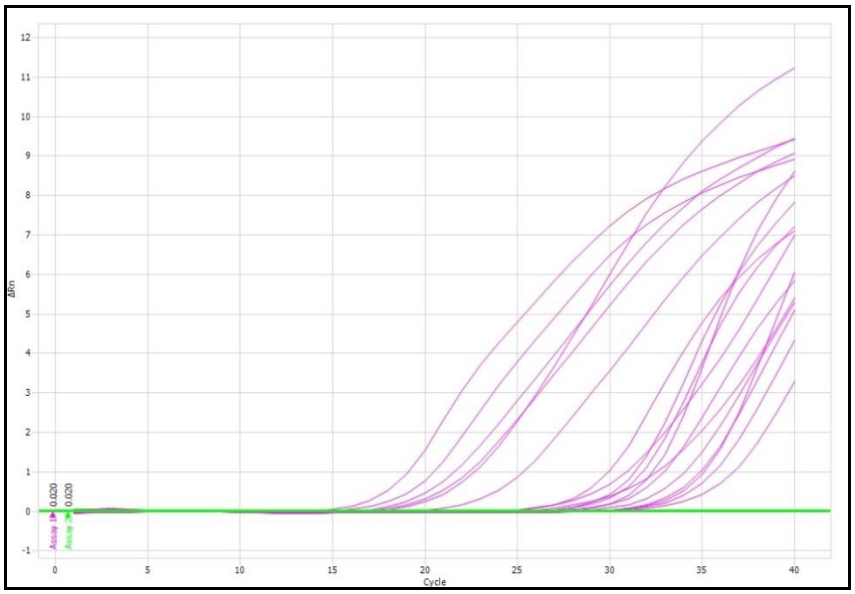

Fig. 3. Amplification plot of real-time PCR assay for *lipl32* gene from clinical samples

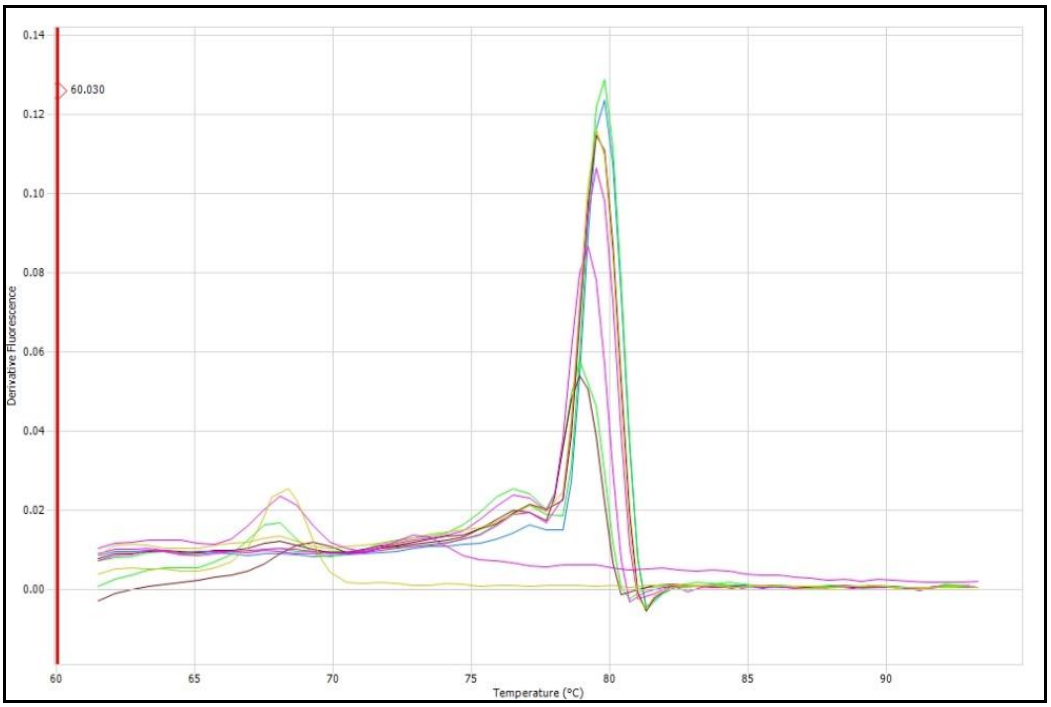

Fig. 4. Melt curve of real-time PCR assay for *lipl32* gene from clinical samples

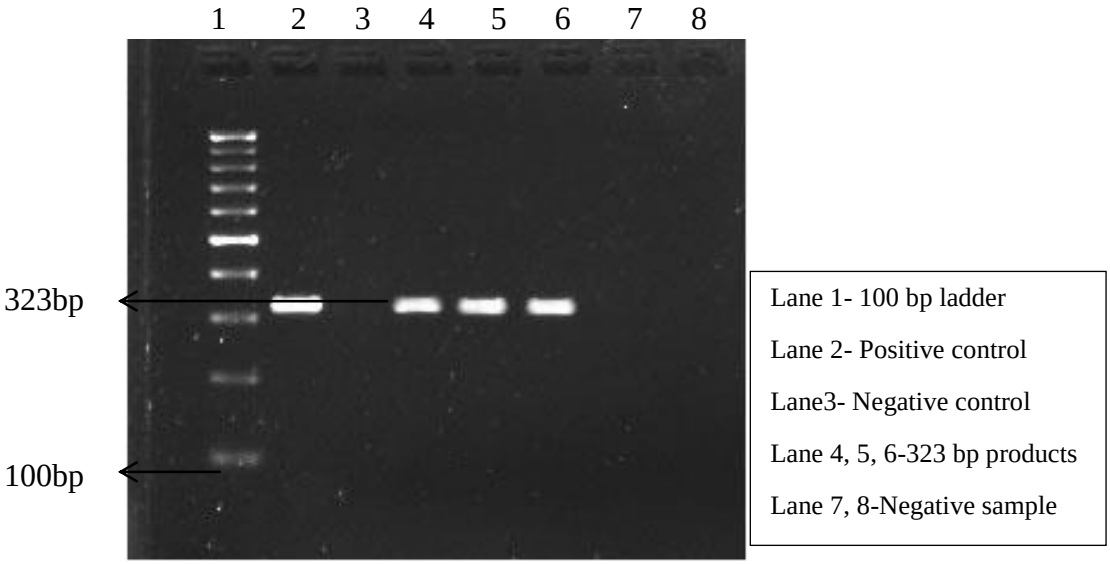

Fig. 5. Agarose gel electrophoresis demonstrating the qPCR products

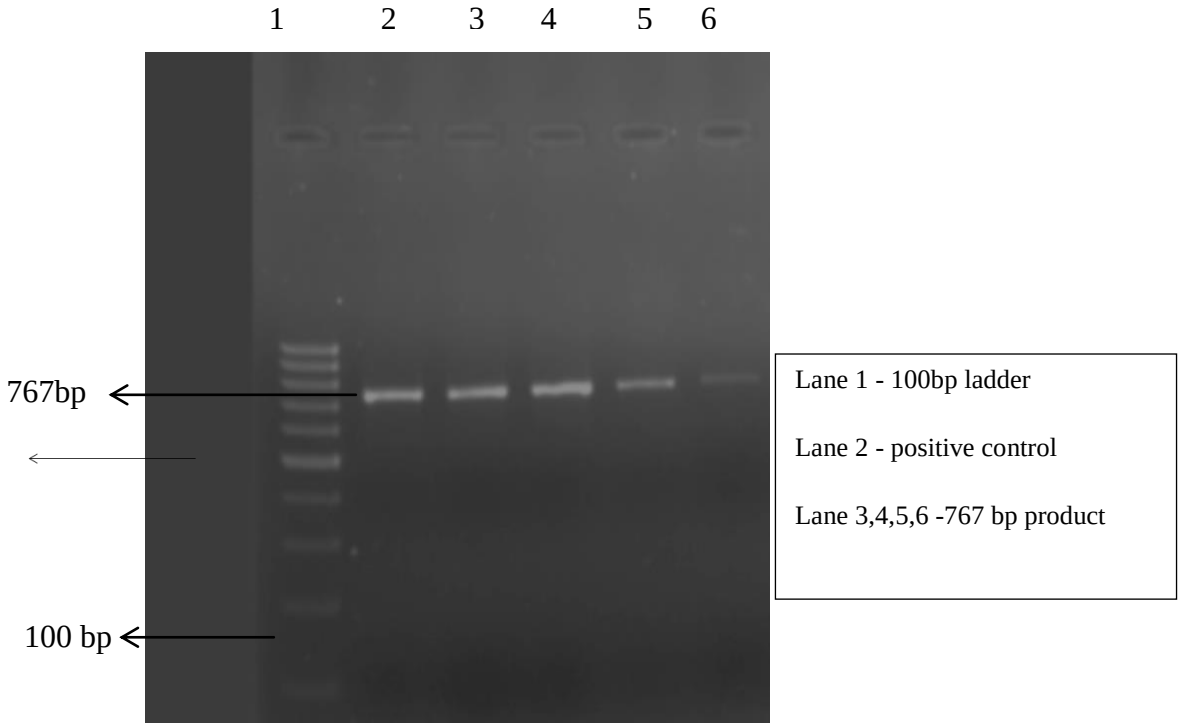

Fig. 6. Agarose gel electrophoresis showing *lipl32* PCR amplicons using the conventional PCR

5. DISCUSSION

Leptospirosis, an anthropozoonotic disease with world-wide distribution is caused by the pathogenic bacteria of the genus *Leptospira.* Considering the higher prevalence and great economical impact, there needs efficient strategies for the effective diagnosis of the disease. The disease affects multi-organ systems and the clinical symptoms resemble other febrile conditions often making the symptomatic diagnosis faulty. Rapid diagnostic aids are hence required for optimal treatment and patient management.

The most common diagnostic tests are direct examination of clinical samples using dark field microscope, isolation of the organisms from clinical samples, detection and quantification of antibodies specific to *Leptospira* using various serological methods and the detection of the organism employing molecular diagnostic tests.

Microscopic agglutination test is the gold standard reference method for the serodiagnosis of the disease. It helps in the identification of the infecting serovars of *Leptospira* and also aids in the epidemiological surveillance. However, it needs a well-equipped laboratory and expert supervision for the performance and interpretation of the results. Inspite of many of its inherent dis advantages, MAT is considered as the most dependable test to confirm cases of individual acute cases. In a disease endemic region like Kerala, a positive result in MAT should be evaluated and interpreted properly because of the presence of antibodies by virtue of past infection or vaccination in canines (Wolff and Bohlander, 1966). The methods for the direct detection of leptospires include DFM, isolation and molecular methods like PCR and real-time PCR. Dark field microscopy is highly insensitive and also nonspecific, it requires 10^4cells per millilitre for the detection of pathogen and many of the serum components may be visualised as artefacts with similar morphological behaviour as that of *Leptospira* (Ahmad *et al.,* 2005). Cultivation of the organisms, though confirmative may take long time so considered as a less preferred choice for acute disease diagnosis. The molecular

methods like PCR are considered to be reliable choice for the detection of relatively smaller number of pathogen in the diagnostic sample. Also, the relatively simple protocol and the quick delivery of the results make them as preferred diagnostics during acute disease case management. Further to increase the efficiency of PCR, real-time PCRs were employed which could be performed in a much faster time than required for the conventional PCRs and moreover, the results can be read directly from the apparatus after the protocol, without the need for further analysis of the results in separate agarose gel electrophoresis as required in the case of conventional PCR. The present study envisages the evaluation of real-time PCR as compared with the conventional PCR and MAT for the detection of acute canine leptospirosis in dogs.

During the study period samples were collected from 75 cases suspected for canine leptospirosis presented to the Teaching veterinary clinical complex Kokkali and Mannuthy. The different samples include blood for sera for to conduct MAT, blood collected in EDTA vials for the performance of PCR and real-time PCR and urine samples were also collected from few of the cases for conducting molecular tests. The sera were subjected MAT at 1:400 dilution and the samples which yielded a titre ≥ 1: 400 above were considered as positive. Similar observations were made earlier Guerreiro *et al*. (2001).

The different infecting serovars identified were Australis 33.33 per cent, Autumnalis 19.44 per cent, Canicola 5.55 per cent, Pomona 8.33 per cent, Icterohaemorragiae and Bataviae with a same percentage of 11.11 and Hebdomadis 8.33 per cent. Similar reports depicting the prevalence of Australis and Autumnalis in canine leptospirosis were reported earlier (Abhinay, 2011). By conducting MAT the specific serovars responsible for the disease could be identified but the challenge was some of the early leptospiral cases were underdiagnosed by MAT because of the absence of the required level of antibodies needed for the agglutination reaction in the patient's serum. In addition, a confirmatory disease diagnosis can be made only by demonstrating a four-fold increase in the titre in paired serum samples collected two to three weeks apart. In

the present study also few of the samples were showing inconclusive results in MAT, which warranted further testing of the sera samples in paired sera collected at two to three weeks apart. Hence, specific and unequivocal results using MAT requires time, making it unfit for acute case management. Above all, MAT being a highly serovar specific test requires the inclusion of a battery of leptospiral serovars prevalent in a particular region to give a confirmatory result and also it makes the test cumbersome to perform. Hence, though MAT is regarded as the gold standard serological test for the confirmative disease diagnosis of leptospirosis, at times, the results may require further confirmation. The best alternatives could be molecular tests like PCR which can yield sensitive, specific and quicker delivery of results. Thus in the present study clinical samples were tested in MAT, followed by PCR and real-time PCR.

From the collected samples, DNA was extracted using a Qiagen DNA extraction kit. This method was reported proven for the preparation of DNA template from whole blood and urine (Karuniawati *et al*., 2012). In the present study, EDTA vials were used to collect the samples and it has given a greater amplification efficiency compared to heparinised and citrate added tubes (Levett *et al*., 2005). Vacutainer tubes containing EDTA gave optimal results up to five days after addition of blood containing viable leptospires; tubes containing heparin were inhibitory as reported previously (Smyth *et al.*, 2002).

The urine samples needed to be processed as early as possible because acidic pH eventually abolishes the organism within the samples. Hence, the urine samples must be diluted with PBS or with any other diluent soon after collection (Ambily, 2012). Here the urine samples were diluted by using PBS and sterile nuclease free water.

For diagnosing the samples using conventional PCR assay, all suspected samples from which the DNA extracted were subjected to PCR using the primers specific for *lipl32*, the outer membrane protein conserved for all pathogenic serovars. Outer membrane proteins were genus specific and possessed high sensitivity and specificity with minimal background reactions and hence were

appropriate antigens to be employed in various serological assays (Srivastava *et al*., 2006, Sharma *et al*., 2007 and Joseph *et al*., 2012). The whole cell leptospiral antigens though were found sensitive for the detection of leptospiral antibodies were demonstrated to have high degree of cross reaction with other Gram negative organisms making it less specific diagnostic candidate and hence were discontinued (Matsuo *et al*., 2000). *Lipl32* is a highly conserved gene in pathogenic leptospires during infection in mammals and it has been identified as an extracellular matrix-binding protein specifically to laminin, collagen I, and fibronectin. It increases the cell permeability and accelerates the apoptosis process. Hence, this gene is selected as our gene of interest for primer designing in PCR as its greater specificity towards the pathogenic serovars.

Out of seventy-five samples, 30 (40 per cent) were recorded as positive in conventional *lipl32* based PCR. Before proceeding on to the performance of PCR on clinical samples, the sensitivity or the detection limit of the assay was determined by testing the tenfold diluted DNA extracted from leptospiral culture. The extracted DNA was diluted in nuclease free water and the concentration was measured in nano-drop. It was determined that the minimum detection limit of the *lipl*32 based PCR was 82.3 ng/ µL. Similar work for calculating the minimum detection limit was performed earlier by Karuniawati *et al.* (2012).

It was observed that the conventional PCR was demonstrated to have 80.5 per cent sensitivity and 97.4 per cent specificity compared to MAT. In the present study the PCR assay could detect six numbers of samples more than MAT. This clearly indicates the relevance of using molecular methods like PCR for the early detection of leptospirosis where MAT fails. Similar results were reported in earlier works (Smyth *et al.,* 2002). However, to know results of PCR, the products needs to be electrophoresed manually and further visualized, which may further delay the delivery of the results. In order to overcome this hurdle, real-time PCR was tested by many researchers (Levett *et al.,* 2005 ; Fearnley *et al.,* 2008). It was reported that real-time PCR generates the results much earlier than conventional PCR and it enables the direct interpretation of the results directly from the

apparatus during the cycle running itself, making it as a more preferred protocol for the quick delivery of the accurate results. Besides, the real-time PCR enables the quantification of leptospires in the clinical samples, thereby enables the identification of the severity of the infection for adopting the best treatment regimen.

Thus in the present study, the next step was to standardise a real-time PCR for the detection of leptospires in the clinical sample. Real-time PCR was standardized using the specific primers of *lipl32* gene with gradients of temperatures using Maxima SYBR Green qPCR master mix (2X). It was observed that an annealing temperature of 60^0C for 30 seconds gave an optimal result. Temperature selection for annealing based on the Tm value and substrate signal (the starting point where the fluorescence is detected) among the tested temperatures. Similar method of optimization of the real-time PCR was reported by Levett *et al.* (2005).

In the present study, for real-time PCR assay, SYBR green chemistry was employed. The advantage of SYBR-green lies in its cost effectiveness when compared to FRET and *Taq* man probes but the limitation is that it is not specific for the target and may give a false positive signal with non-specific amplicons (Smythe *et al.*, 2002). However, the primers when tested with extracted DNA from *Leptospira* could yield only a single amplicon, specific with desired size, when tested on agar gel electrophoresis (Fig. 5). Further, on melt curve analysis (Fig. 2), a single peak corresponding to a single amplicon was observed. Thus, it was confirmed that the primer used in the study was sensitive and specific. Subsequently, the minimum DNA detection limit was determined in real-time PCR and it was observed to be 9 ng/ µL. This clearly indicates the improved sensitivity of real time PCR than the than conventional PCR for the detection of leptospiral DNA.

The total volume of the real-time PCR assay was 12.5 µL, in which 6.25 µL of the 2X master mix containing the polymerase and dNTPs in the optimized buffer. The DNA extracted from cultures was kept as positive control and negative control was made with nuclease free water. An amplification plot was

drawn and it showed a Tm value of 79.79. Further, melting curve was analysed for the tested samples, which was almost observed at the 79.79 (Fig. 2).

Real-time PCR is found to be a promising, rapid and appropriate diagnostic test to aid in clinical case investigation. The advantage of using real-time PCR over conventional PCR is that, the amplification is completed in less than an hour and the assay is less sensitive to contamination (Picardeau, 2014). According to Smythe *et al*. (2002), when compared to culture, real-time PCR requires only two cells for detection. Some of the validated studies showed that real-time PCR for detecting pathogenic leptospires has given an analytical sensitivity up to 3 genome equivalents per reaction in blood and 10 genome equivalents in urine (Levett *et al*., 2005). Real-time having a high throughput sequence detection system, we can analyse the samples on a large scale compared to conventional techniques.

The sensitivity, specificity and accuracy of conventional and real-time PCR assays were found out in comparison with MAT. In conventional assay, among the 75 serum samples examined, 30 (40 per cent) were positive. Thirty-six samples were found to be positive in MAT at 1:400 dilutions. The sensitivity, specificity and accuracy of PCR when compared to MAT was 80.55, 97.4 and 89 per cent respectively. In real-time assay, among the 75 serum samples examined, 39 (52 per cent) were positive and in MAT 36(48 per cent) were positive. The sensitivity, specificity and accuracy of real-time PCR in comparison to MAT was 91.6, 84.61 and 88 per cent, respectively.

For the comparison of MAT, PCR and real-time PCR, Chi square test was used. Among these three methods, real-time PCR is having a higher sensitivity and specificity compared to other two. For the detection of antibodies against *Leptospira*, either ELISA or MAT is used, but lack of sufficient antibodies in patient's sera in the early phase of infection result in an inability to confirm the diagnosis. Real-time PCR assay provides the potential to detect pathogenic *Leptospira* spp. in a range of clinical specimens providing an earlier diagnosis and unequivocal evidence of active infection.

To conclude, real-time PCR can be applied for the rapid detection and quantification of leptospires in biological samples and compared with other assays, it is highly sensitive and specific. Even though the test is costly, it could be an effective tool and a revetment to conventional methods to ensure an accurate and timely diagnosis of leptospirosis, especially in endemic areas. The technique needs to be more redefined, so that in future it could be used as a valuable adjunct for the diagnosis of leptospirosis in veterinary field.

6. SUMMARY

Leptospirosis is a fatal zoonotic disease prevalent in all most all countries in the world. In many of the places in south India, especially Kerala is known to be one of the endemic areas. The identification of the disease in its early stage is important for the initiation of appropriate treatment as well as patient management is concerned. The diagnosis of the disease is primarily based on direct detection of the organism using dark field microscopy, detection and quantification of antibodies using serology and molecular tests. In order to overcome the limits of the conventional methods and to find a remedy for the acute disease case management molecular techniques such as PCR could be an efficient tool for early diagnosis of the disease, especially when the clinical expression of the disease is confusing. Limitation in the PCR is that for the confirmation it requires an agarose gel electrophoresis, which will further be delaying the result delivery. So in order to make a result delivery faster, real-time PCR was tested by many of the researchers working in diagnostic microbiology. Conventional PCR could detect the disease at the end of the reaction cycling only, whereas the real-time can monitor each and every step of the reaction and can read the results directly from the apparatus during the running time itself. The present study aims to evaluate the diagnostic efficiency of real time PCR in comparison with the conventional technique to evaluate its suitability in the acute disease case detection in a leptospirosis endemic region.

In this study a total seventy-five sample suspected for canine leptospirosis were screened using the various diagnostic tools such as microscopic agglutination test, PCR and Real-time PCR.

A total of eight serovars prevalent in our area were used as the antigens. MAT was performed according to the regulations given by OIE (2008) at 1:400 serum dilution. A total of 36 samples had shown a titre of ≥1:400 were taken as positive.

For performing the conventional *lipl32* based PCR, DNA was isolated using the DNA isolation kit and the concentration was checked using the nano-drop spectrophotometer. The minimum detection limit of PCR was found out using the

tenfold diluted DNA extracted from the live leptospiral culture as template. The minimum detection limit was observed to be 82.3ng/µl. Out of 75 samples tested, 30 were shown to be positive for PCR. The sensitivity, specificity, and accuracy of PCR were 80.55, 97.4 and 89 per cent, respectively in comparison with MAT.

A *lipl32* based real-time PCR was also standardized which were optimised at an annealing temperature of 60ºC for 30 seconds. The minimum detection limit of real-time was detected as 9 ng/ µl. A total of 39 samples (52 per cent) were detected as positive with the real-time PCR. The sensitivity, specificity and accuracies were compared with that of MAT and shown a percentage of 91.6, 84.61 and 88, respectively.

Thus it is made clear that the real-time PCR could be a promising, rapid and effective technique for the pathogen detection in acute clinical cases where other tests give ambiguous or false negative results. This could be attributed to its very low DNA detection limit as observed in the study, which ensures the detection in less concentrated samples. Though the high cost of the performance of real time PCR may limit its routine use for sample testing, it can be kept as an alternative diagnostic step, where the other tests like MAT and conventional PCR gives false results inspite of the classical clinical leptospirosis symptoms in acute disease cases.

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REAL-TIME PCR BASED DETECTION OF LEPTOSPIRES IN BLOOD AND URINE OF DOGS

SUBI T. K.
(14-MVM-35)

ABSTRACT

Submitted in partial fulfilment of the requirement for the degree of

MASTER OF VETERINARY SCIENCE

(Veterinary Microbiology)

2016

Faculty of Veterinary and Animal Sciences
Kerala Veterinary and Animal Sciences University

DEPARTMENT OF VETERINARY MICROBIOLOGY
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY, THRISSUR-680 651
KERALA, INDIA

ABSTRACT

Leptospirosis is one of the most widespread zoonoses. The early diagnosis of the disease based on the symptoms is often difficult because of its overlapping symptoms with many other febrile illnesses. The present study envisages the evaluation of real time PCR and conventional PCR as compared to the Microscopic Agglutination Test (MAT), for the detection of acute canine leptospirosis. A total of 75 samples were collected from the cases suspected for canine leptospirosis, which include sera for conducting MAT, whole blood for PCR and Real time PCR and urine samples. The sensitivity, specificity and accuracy of conventional and real time PCR assays were determined keeping MAT as the reference. When the sera samples at 1:400dilution were tested in MAT, 36 of them were found to be positive. The sensitivity, specificity and accuracy of the conventional PCR when compared to MAT was 80.55, 97.4 and 89 percent, respectively. Similarly the values observed with real time PCR vs MAT were 91.6, 84.61 and 88 percent, respectively. From the results it could be concluded that real time PCR assay standardized in the present study was found to be more sensitive than the conventional PCR. In addition, the real time PCR assay could deliver the results quickly and enable quantification of the organisms in the sample, which would aid in much more efficient case management.

Acad. Form No.19

KERALA VETERINARY AND ANIMAL SCIENCES UNIVERSITY

Faculty of Veterinary and Animal Sciences

PROGRAMME OF RESEARCH WORK FOR THESIS FOR MASTER'S DEGREE

1. Title of thesis

Real-time PCR based detection of leptospires in blood and urine of dogs

2. (a) Title of the departmental/KVASU research project of which this forms a part

Not applicable

(b) Code no. if any, and order by which the departmental/KVASU research project is approved

Not applicable

3. a) Name of student

Subi T.K.

b) Admission No.

14-MVM-35

4. a) Name of Major Advisor (Guide)

Dr. Siju Joseph

b) Designation

Assistant Professor,
Department of Veterinary Microbiology,
College of Veterinary and Animal Sciences, Mannuthy- 680 651

5. Objectives of the study

1. To determine the minimum detection limit of the leptospiral DNA using conventional and real time PCR
2. To standardize real time PCR for the detection of leptospiral organism in canine blood and urine

6. Practical/Scientific utility

Definitive diagnosis of leptospirosis relies on isolation of leptospires or demonstration of seroconversion in paired sera. However, both these methods are time consuming and hence could not be of much use in acute disease diagnosis. Hence, alternative approach likes PCR was attempted and proved as a rapid and efficient diagnostic. In order to augment the diagnostic efficacy, real time PCR was tried for the detection

and quantification of leptospiral DNA from the clinical samples. However, its efficacy in detecting leptospiral DNA from canine clinical samples remains unexplored. Hence, the present study envisages the standardization of real time PCR for the detection of leptospiral DNA from canine blood and urine samples.

7. Important publications on which the study is based

Microscopic Agglutination Test (MAT) is the gold standard confirmatory test, most likely based on the sero reactivity with lipopolysaccharide antigens. (Faine, 1982).

Real time PCR assays are genus-specific and detect all leptospiral serovars, both pathogenic and non-pathogenic as proved by Merien *et al.* (1992).

Haake *et al.* (2000) developed a real-time assay using SYBR Green chemistry, in which the target was the gene for the major outer-membrane lipoprotein *Lipl*32.

Smythe *et al.* (2002) reported the utility of real time PCR based on TaqMan probes for the diagnosis of leptospires in clinical and environmental samples and observed a very low detection limit as low as two cells in the sample.

Levett *et al.* (2005) detected pathogenic leptospires by real time quantitative PCR based on SYBR Green fluorescence.

Merien *et al.* (2005) developed a cost effective SYBR Green based real time PCR assay for the detection of pathogenic leptospires in human sera and observed a sensitivity of 50 leptospires/ mL of the sample.

Palaniappan *et al.* (2005) evaluated *lig* gene based real time PCR for the detection of pathogenic leptospires.They concluded that the real time assay as a rapid and sensitive diagnostic method as compared to culture and conventional PCR.

The efficacy of primers capable of amplifying conserved outer membrane protein genes *lipl32* and *lipl41* of *Leptospira* was successfully tested by Cheema *et al.* (2007).

Karuniawati *et al.* (2012) optimized a real-time PCR to detect *Leptospira* spp. in human blood and urine specimens and observed that the minimum limit of detection for DNA was 150 fg/µL and 1470 fg/µL from blood and urine, respectively.

Ahmed *et al.* (2012) validated real time PCR based on SYBR Green chemistry for the detection of leptospires from serum samples collected from experimentally infected dogs. They observed a sensitivity and specificity of 91.7 per cent and 90 per cent, respectively.

Agampodi *et al.* (2012) reported that, despite the cost and logistical challenges, real time PCR has the potential to provide accurate and timely diagnosis for human leptospirosis at the point of care in endemic areas.

8. Outline of the technical programme

Clinical samples including sera, whole blood and urine will be collected from a minimum of 75 suspected case of canine leptospirosis presented to Teaching Veterinary Clinical Complex at Mannuthy and Kokkalai.

The collected sera will be screened using only MAT to identify the infected animals (Faine, 1982).

Primers for *lipl32* gene of *Leptospira* will be designed for conventional PCR and SYBR Green chemistry based Real time PCR. The protocol will be standardized respectively.

The DNA will be extracted from leptospiral organism, concentration will be determined using nanodrop method and different dilutions of DNA will be subjected to PCR and real time PCR for determining the minimum detection limit

The specificity of the real time PCR assay will be determined by testing the extracted DNA from bacteria other than *Leptospira.*

The DNA from the clinical samples will be extracted and subjected to conventional and real time PCR assays.

The data obtained from MAT, conventional PCR and real time PCR will be compared, analyzed and results will be interpreted.

9. Main items of observations to be made

1. Minimum detection limit of leptospiral DNA in conventional and real time PCR.
2. Sensitivity and specificity of conventional PCR and real time PCR with respect to MAT.
3. Relative efficacies of conventional and Real time PCR.

10. Facilities

(a) Existing

Work will be commenced with existing facilities available in the Department of Veterinary Microbiology and other departments of College of Veterinary and Animal Sciences, Mannuthy.

(b) Additional facilities required

(i) Chemicals
(ii) Biologicals

11. Duration of study

Four semesters

12. Financial estimate

Cost of chemicals and Biologicals	: Rs.20, 000
Contingencies	: Rs.5, 000
Total	: Rs.25, 000

Signature of student

Signature of Major Advisor

Place: Mannuthy
Date: 5.08.2015

Name, Designation and signature of members of the Advisory Committee

Chairperson

Dr. Siju Joseph,
Assistant Professor,
Department of Veterinary Microbiology,
College of Veterinary and Animal Sciences,
Mannuthy, Thrissur- 680 651.

Members

Dr. M. Mini,

Professor and Head,
Department of Veterinary Microbiology, College of Veterinary and Animal Sciences,
Mannuthy, Thrissur-680 651.

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APPENDIX-I

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APPENDIX II

Time frame of work

Semester I

1. Collection of literature.
2. Preparation of research proposal.
3. Maintenance of the *Leptospira* isolates maintained in the Department.of Veterinary Microbiology
4. Collection of sera from dogs suspected to have leptospirosis.
5. Screening the sera using MAT

Semester II

1. Collection of sera and MAT continued.
2. Standardization of conventional and real time PCR
3. Determination of the DNA detection limits in both PCR assays

Semester III .

1. Checking the specificity of the PCR assays.
2. Testing the DNA extracted from the clinical samples using conventional and real time PCR assays

Semester IV

1. Comparing the results obtained from MAT, PCR & real time PCR.
2. Analysis and interpretation of results
3. Submission of thesis

CERTIFICATE

Certified that the research project has been formulated observing the stipulations laid down under the Prevention of Cruelty to animals Act (Amendment, 1998).

Place: Mannuthy **Dr. Siju Joseph**

Date: Major Advisor

CURICULUM VITAE

Name of candidate	: SUBI T.K.
Date of birth	: 14/11/1989
Place of birth	: Adoor, Pathanamthitta, Kerala
Marital Status	: Single
Permanent Address	: D/O Kuttan M. K. Subinbhavanam, Anayadi P.O, SooranaduNorth, Kollam , Kerala
Major field of specialization	: Veterinary Microbiology
Educational status	: B.V.Sc & A.H, undergoing M.V.Sc
Professional Experience	: Veterinary Surgeon as contract
Accepted article for Publication	: Standardization of real-time PCR for the acute canine leptospirosis (Journal of Veterinary and Animal Sciences)
Membership in professional societies	: Indian Veterinary Association Kerala State Veterinary Council