STANDARDISATION OF REAL-TIME PCR FOR THE DETECTION OF ACUTE CANINE LEPTOSPIROSIS

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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 1:400 dilution 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 for 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.

Key Words: *Leptospirosis; Real-Time PCR; Amplification plot, Melt curve*

Leptospirosis is a worldwide under diagnosed disease caused by spirochaetes belonging to the genus *Leptospira*. Definitive diagnosis of the disease is carried out by detection of the organisms in clinical specimens such as blood or urine, cultural isolation(Faine, 1982), molecular methods for the direct detection of leptospires or by serological tests that detects leptospiral antibodies (Levett, 2003).However all these methods are cumbersome and time consuming. The molecular diagnostic techniques developed so far are mostly based on polymerase chain reaction (PCR) assays, which requires well-established laboratory facilities and highly

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skilled personnel. Even though the direct detection of leptospires from clinical samples were successfully accomplished by Polymerase chain reaction (PCR), the interpretation of the results require further analysis on agarose gel electrophoresis.In order to circumvent the disadvantages of conventional PCR, real time PCR was carried out. The procedure follows the general principle of polymerase chain reaction, but its key feature is that the amplified DNA is detected during the progression of the reaction itself and enables the quantification of the target DNA, which would aid in understanding the gravity of infection.This assay has the potential to facilitate rapid, sensitive diagnosis of acute leptospirosis. 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(Agampodi 2012). The present study envisages the standardization of a Real-time PCR, to find out its minimum detection limit of DNA in the Real-time and conventional PCR and the sensitivity specificity and accuracy of Real-time assay in comparison with the MAT.

Materials and Methods

Collection of samples

A total of 75 sera samples, whole blood and urine were collected from cases suspected for canine leptospirosis presented to the teaching veterinary clinical complex, Kokkali and Mannuthy. The different samples such as sera for conducting MAT, Blood collected in EDTA vials and urine samples for the performance of PCR and Real-time PCR

Extraction of DNA

The DNA was extracted from 200 µL of EDTA anti coagulated whole blood samples and reference strains using qia-amp blood DNA extraction kit (QIAGEN, Hildan, Germany) according to the manufacturer's instruction. It was quantified using nanodrop DNA quantifier –(Thermoscientific,USA 2000C model) and stored at -20^{0}C until further used.

Microscopic Agglutination Test

The MAT was carried out using live *Leptospira* organisms following OIE (2008) in a 96 well 'U' bottom microtitre plate. 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. MAT was carried out at a serum dilution of 1:400. Agglutination showing≥1:400 dilution were considered as positive.

Polymerase Chain Reaction

The primers used for the amplification of *lipl32* gene (Cheema *et al*., 2007) of leptospires with following sequences

Forward primer (F1):
5'-CGCGGTCGACGCTTTC
GGTGGTCTGCCAAGC3'

Reverse primer (R1):
5'-CGCGCTGCAGTTAC
TTAGTCGCGTCAGAAG-3'

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 10MmdNTP 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 thermocycler (Eppendorf Mastercycler) for amplification.The amplification were carried out in thermocycler with an initial denaturation at 94^{o}C for four minutes, followed by the sequence of 35 cycles as follows: 94ºC for 1 min, 60ºC for 45 sec. and 72ºC for 2 min. This was followed by final extension at 72ºC for ten minutes. The PCR product was detected by electrophoresis at 50 V and 16 mA in one per cent agarose gel in Tris Acetate EDTA buffer (1X).

Real-time PCR

Primers for real-time PCR of *lipl32* genes as quoted by (Levett *et al*., 2004) were

custom synthesized (Sigma-Aldrich, Bangalore) and obtained in lyophilised form. The primer sequence is listed below:

LipL32 F1-5'CATTCATG TTTCGAATCATTTCAAA3',

LipL32R1-5'GGCCCAAGTTCCTTCTAAAAG3',

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.The DNA extracted from cultures was kept as positive control and negative control was made 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. The amplification were carried out in thermocycler (Applied Bio system step one plus) with an initial denaturation at 94°C for four minutes, followed by the sequence of 40 cycles as follows: 94ºC for 30 sec, 60ºC for 30 sec. Finally, an amplification plot and melt curve were analysed for the result confirmation.

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

The detection limit for PCR and Real-time PCR were estimated using DNA extracted from standard strain of *Leptospira*serovar Australis culture. The DNA was serially diluted to 10 fold for real-time PCR. The amplification plot showed cycle number on the X axis and Delta Rn (Fluorescence) on the Y axis 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.

Results and Discussion

A total of 75 canine sera 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. Out of this 30 DNA samples could be detected positive using conventional PCR. The sensitivity, specificity and accuracy of PCR relative to MAT was calculated to be 80.55 per cent, 97.4 per cent and 89 per cent, respectively (Table no.1). Minimum detection limit of DNA in PCR was calculated as 82.3ng/µl.The results of the Real time PCR were also compared with that of MAT. Among the 75blood samples examined, 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 (table no.2) to be 91.6per cent, 84.61 per cent and 88 per cent, respectively. In the present study of 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 (Smyth *et., al* 2002). However, the primers when tested with

Table 1. 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%
Specificity = 97.4%
Accuracy = 89%

Table 2. 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 = 91.6%
Specificity = 84.61%
Accuracy = 88%

extracted DNA from *Leptospira* could yield only a single and specific amplicon of 323 bp size, when tested on agar gel electrophoresis (Fig.1). 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. The minimum DNA detection limit was determined in real- time PCR and it was observed to be 9 ng/µl. 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 compared to other two. 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 (Ahmed 2012).

To conclude, real-time PCR could be employed for the rapid detection and quantification of leptospires from biological samples, compared with other assays, it is highly sensitive and specific. Even though the

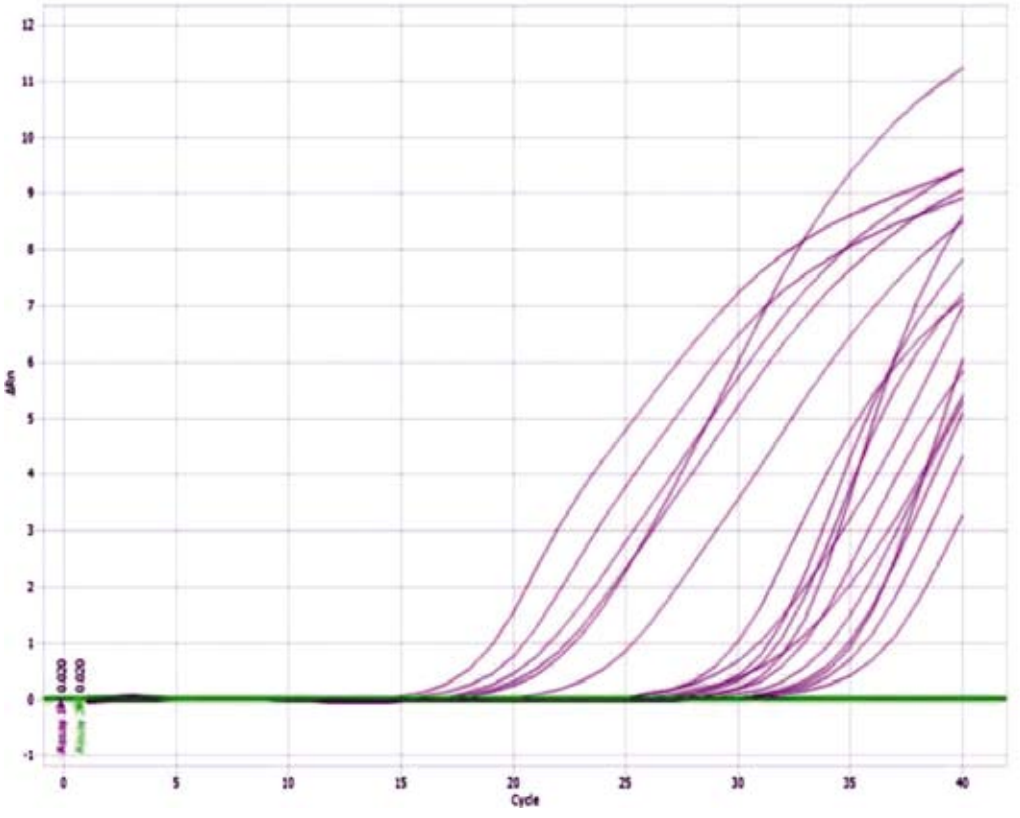


Fig. 1. Amplification plot of real-time PCR assay for *lipl32* gene from clinical samples

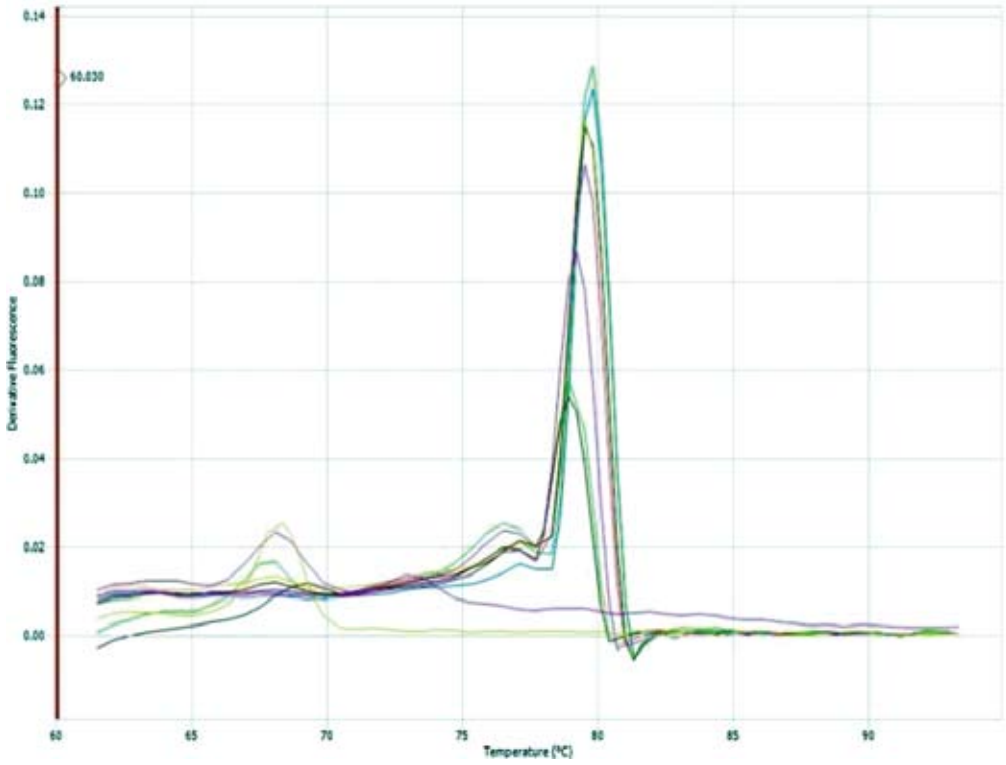
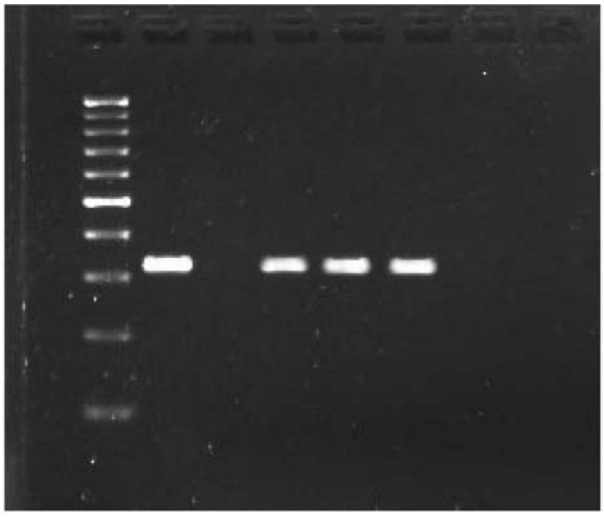


Fig. 2. Melt curve of realtime PCR assay for *lipl32* gene from clinical samples



Lane 1- 100 bp ladder
Lane 2- Positive control
Lane3- Negative control
Lane 4,5,6-323bpproduct
Lane 7,8-Negative sample

Fig. 3. Agarose gel electrophoresis showing *lipl32* PCR amplicons using the real-time PCR

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.

From the current study we could standardise a real-time PCR for the detection of leptospires from the acute canine leptospirosis cases. The minimum detection limit of PCR and real-time was PCR 83.9ng/µl and 9 ng/ µlrespectively andthe sensitivity, specificity and accuracy of PCR as relative to MAT was

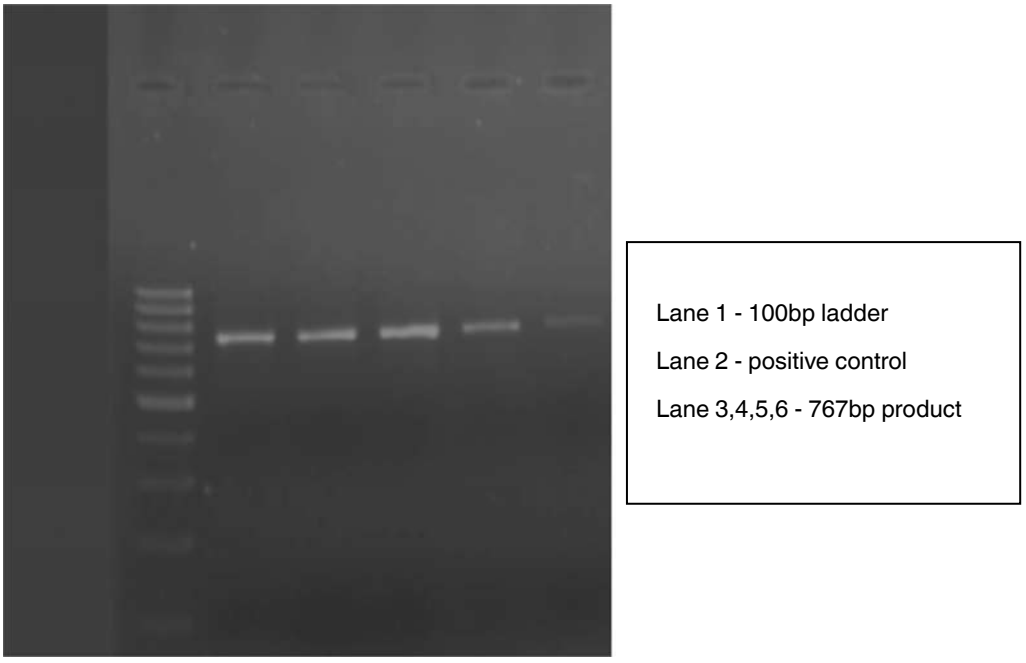


Fig. 4. Agarose gel electrophoresis showing *lipl32* PCR amplicons using PCR. \

calculated to be 80.55 per cent, 97.4 per cent and 89 per cent, respectively. Among the 75 samples 39(52%) 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 91.6per cent, 84.61 per cent and 88 per cent, respectively.

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