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Taqman based real time polymerase chain reaction (qPCR) for detection of *lipL32* gene of pathogenic leptospires from human, canine and feline blood samples for diagnosis of acute infections and study carrier status

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Abstract
Leptospirosis is a zoonotic disease caused by bacterium belonging to genus *Leptospira*. Domesticated animals like dogs, cats as well as human beings can get infected with the disease. Signs and symptoms can range from mild (headaches, muscle pains and fevers) to severe symptoms like bleeding in lungs or meningitis. The acute severe form of disease called Weils disease-causes the infected individual or animal to become jaundiced, develop kidney failure and death within days. Laboratory diagnosis of leptospirosis under field conditions is very difficult and depends mainly on indirect antibody detection tests like Microscopic Agglutination Tests (MAT) or IgM/IgG one step ELISA tests. MAT is difficult to perform under filed conditions and it requires labs with sophisticated infrastructure to maintain live cultures of *Leptospira*. During acute phase of disease-MAT is not specific because of cross reactivity between serovars. The aim of present study is to standardize a Real Time (qPCR) protocol based on taqman chemistry for rapid diagnosis of pathogenic leptospires in humans, canines and felines EDTA blood samples collected during acute or febrile phase where there is most likely chance of leptospires in blood (bacteremic phase). RT PCR will also be used to identify carrier status in dogs considered as clinically normal but possibility of environmental exposure to pathogenic leptospires or animal may be suffering from undetected or subclinical infection as evident from the detection of *lipL32* gene of pathogenic leptospires in EDTA blood.

Keywords: Leptospirosis, dogs, cats, humans, real time PCR, lipL32 gene

1. Introduction
Disease leptospirosis caused by spirochete belonging to genus *Leptospira* has emerged as a serious global public health problem. Leptospirosis is a major threat to state of Kerala with more than 1000 cases being reported annually (As per Integrated Disease Surveillance Project-IDSP- report of Directorate of Health Services, Kerala). The disease often goes undiagnosed and is endemic in many places of South India with 1745 confirmed cases and 97 deaths reported during 2021 in the state of Kerala. The pathogenic leptospires are divided into more than 250 pathogenic serovars with closely related serovars grouped into 26 pathogenic sero groups. Vaccines currently available for dogs gives protection only against 4 serovars *viz*; *L. canicola*, *L. grippotyphosa*, *L. icterohaemorrhagiae* and *L. pomona*. Despite routine vaccinations there are many clinically confirmed cases of leptospirosis reported among domesticated canine population. Laboratory diagnosis of leptospirosis under field conditions is very difficult and practicing veterinarians mainly rely on clinical signs exhibited by animals. In majority of cases initiating treatment after laboratory confirmation is time consuming because diagnostic tests currently used are based on antibody detection-one step rapid tests-like IgM/IgG ELISA or MAT. MAT is difficult to perform under field conditions where serial dilutions of patient sera has to be mixed with different serovars of *Leptospires*-and requires a sophisticated laboratory setting to maintain a panel of live bacterial cultures as antigens. The aim of present study is to standardize a rapid-taqman based RT PCR protocol for qualitative detection of pathogenic leptospires in human, canine and feline EDTA blood samples collected during acute or febrile phase and also to study carrier status among dogs not exhibiting clinical signs.

With this standardized PCR protocol results can be generated in the laboratory within 3-4 hours (all steps including template preparation and PCR test proper).

2. Materials and Methods

2.1 Samples

Whole blood samples in EDTA collected during febrile phase from two human patients dead after showing all classical symptoms of leptospirosis including hematological abnormalities, received from Government Medical College Hospital (GMCH), Thrissur and from sixteen dogs presented to clinical laboratory with clinical signs highly suggestive of leptospirosis *i.e* rise in temperature, congested mucus membrane, ascites and icterus and two cats presented with clinical signs suggestive of cystitis with fever, anorexia, voiding of urine mixed with blood for past few days-formed the material for study and standardization of experiments. Whole blood samples in EDTA were also processed as templates from 95 dogs which were not suspected initially for leptospirosis but were showing clinical signs suggestive of various tick transmitted diseases at the time of sampling-to study the carrier status of pathogenic leptospires if any.

2.2 DNA extraction

Peripheral blood in EDTA was stored at-80°C until usage and processed for preparation of template DNA as per the protocols described in High Pure PCR template preparation kit from blood (Version 27-Roche Diagnostics). The DNA templates prepared from whole blood were stored at -80°C until usage.

2.3 Estimation of concentration of Template DNA

The DNA templates prepared from whole blood were processed for estimation of DNA concentration as per protocols described for (Invitrogen-Qbit ds DNA High Sensitivity Assay Kit) and estimated in Qbit.3.0 Fluorometer (Thermo Fischer Scientific, USA).

2.4 Primers and Probes for PCR

Two pairs of forward, reverse, sense primers and probe sequences targeting pathogenic *Leptospira* species major outer membrane protein (*lipL32)* previously published [1] were used in the study with minor modifications. Primers targeting *lipL32* gene-which produces a 241 bp amplicon with a Taqman probe labeled at the 5' and 3' ends with dye FAM (5'Caboxy Fluorescein) and BBQ respectively were used (Table.1)

Table 1: Details of primers and probes used for qPCR

Oligo Name	Reference	Product Size	Target Genome
Lepto2_F, Lepto2_S, Lepto2_R Lepto2_P	Bourhy *et.al*., 2011 [1]	241 bp	*lipL32*

2.5 Optimized conditions for Real Time PCR

Format for Real Time PCR followed–with total reaction volume of 20 µl, using Fast Start Essential DNA Probes Master 2X concentration-10µl, 1µl each of forward, sense and reverse primer (10µM), 1 µl of probe (10µM), 2 µl of water and 4 µl of template DNA extracted from whole blood collected during febrile/bacteremic phase. Negative and positive controls were included in all the runs. *Leptospira* species major outer membrane protein (*lipL32*) positive control was prepared by custom synthesizing entire sequence of specific region of *lipL32* gene and cloned into *pUGM* Plasmid (Sci Genom Labs PVT LTD, Kakkkand, Cochin). Custom synthesized positive clone in *pUG*M plasmid was used for primer efficiency testing and standardization of cycling conditions, optimal for detection of *lipL32* gene from suspected samples. Five (5µl) of clone (initial concentration-10ng/µl) was diluted to serial one in tenths concentration and 5µl from each 1:10 dilution was used as positive control in reaction mixture. Six serial dilutions in duplicate were prepared for the experiment and all the dilutions were tested in RT PCR assay to find out the limit of detection and efficiency of the primers designed. Cycling conditions optimized for primer efficiency testing and for testing of samples after standardization with positive control, included an initial activation (preincubation) at 95 °C for 10 min followed by 40 cycles of 15s denaturation at 95 °C, followed by a 1 min annealing -extension step at 60 °C.

3. Results

3.1 Estimation of concentration of Template DNA

DNA concentrations were estimated for all the template DNA samples prepared from whole blood prior to Real Time PCR experiments.

3.2 Primers and Probes and Optimized conditions for Real Time PCR

Real Time PCR for amplifying specific region of the *lipL32* gene of *Leptospira* was used for qualitative detection of target genome in blood samples collected from two dead human patients, 16 dogs showing all classical clinical signs of leptospirosis, 2 cats with cystitis and 95 dogs with clinical signs suggestive of various tick transmitted diseases and undergoing treatment. The cycling conditions followed was optimal and successfully amplified *lipL32* gene with specific primers along with taqman probe Lepto 2_P and Cq values less than 40 is considered as positive amplification (Fig. 1). Out of 2 dead human patients, 16 clinically ill dogs and 2 clinically suspected cats, all came out to be positive and treatment was initiated immediately. Out of 95 dogs 44 (44/95-46%) dogs were harboring *lipL32* gene indicating exposure to pathogenic leptospires previously- in a good number of animals even though they are showing clinical signs suggestive of tick borne infections. These animals where undergoing treatment for tick transmitted diseases leptospirosis might had gone undiagnosed, since many at times clinical signs of leptospirosis overlap and get confused with other vector borne illnesses. Amplified products of human samples after sequencing and identity checking with NCBI Blast showed 93% identity to *Leptospira interrogans serovar hardjo* sequences and two canine amplified products showed 97% identity to *Leptospira interrogans serovar hardjo* (Fig.3).

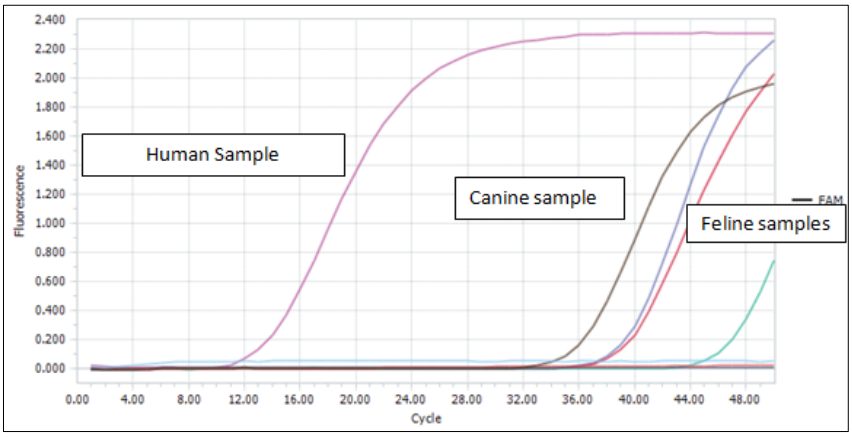


Fig 1: Amplification curve of positive samples of *lipL32 human, canine and feline samples*

3.3 Primer efficiency testing

Primer efficiency testing was conducted by plotting a standard curve, custom synthesized positive clone in *pUGM* plasmid, with initial concentration 10ng/µl was used as template for the plotting of standard curve. Six tubes in duplicate with serial 1:10 dilutions prepared for the experiment showed, change of approximately 3.3 cycles between 10 fold dilutions of the template. The slope of the standard curve generated after primer efficiency testing showed an efficiency of 2.04 and R value 0.98 (Fig.2).

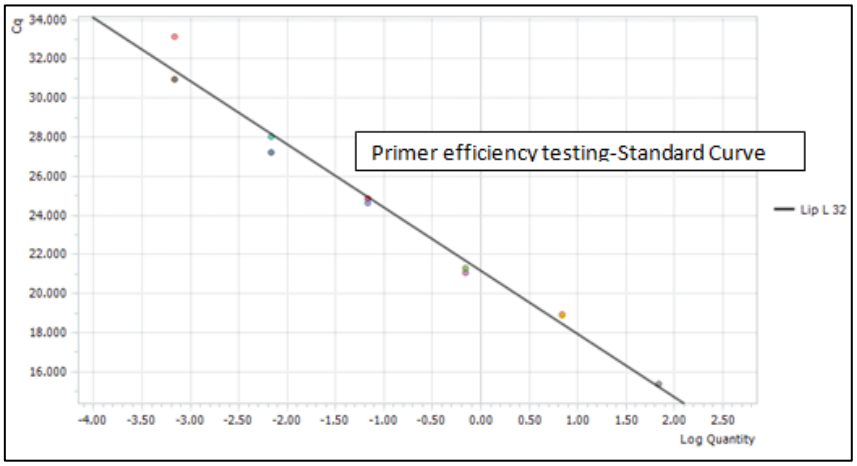


Fig 2: Slope of Standard Curve -Primer efficiency

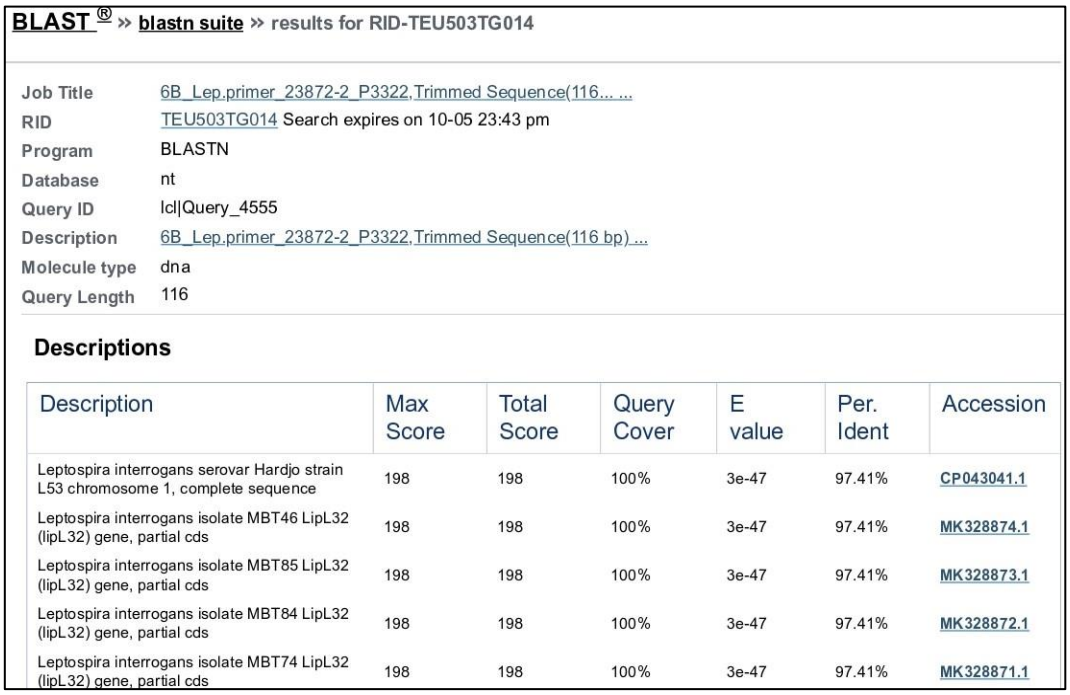
Gene Name	Slope	Efficiency	R^2	Y Intercept
18SrRNA	-3.2382	2.04	0.98	21.14

BLAST® » blastn suite » results for RID-TEUB51KT014

Job Title	2A_Lep.primer_23872-3_P3322,Trimmed Sequence(168... ...
RID	TEUB51KT014 Search expires on 10-05 23:46 pm
Program	BLASTN
Database	nt
Query ID	lcl|Query_196003
Description	2A_Lep.primer_23872-3_P3322,Trimmed Sequence(168 bp) ...
Molecule type	dna
Query Length	168

Descriptions

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Leptospira interrogans serovar Hardjo strain L53 chromosome 1, complete sequence	250	250	100%	1e-62	93.49%	CP043041.1
Leptospira interrogans isolate MBT46 LipL32 (lipL32) gene, partial cds	250	250	100%	1e-62	93.49%	MK328874.1
Leptospira interrogans isolate MBT85 LipL32 (lipL32) gene, partial cds	250	250	100%	1e-62	93.49%	MK328873.1
Leptospira interrogans isolate MBT84 LipL32 (lipL32) gene, partial cds	250	250	100%	1e-62	93.49%	MK328872.1
Leptospira interrogans isolate MBT74 LipL32 (lipL32) gene, partial cds	250	250	100%	1e-62	93.49%	MK328871.1



BLAST ® » blastn suite » results for RID-TEU503TG014

Job Title	6B_Lep.primer_23872-2_P3322,Trimmed Sequence(116... ...
RID	TEU503TG014 Search expires on 10-05 23:43 pm
Program	BLASTN
Database	nt
Query ID	lcl|Query_4555
Description	6B_Lep.primer_23872-2_P3322,Trimmed Sequence(116 bp) ...
Molecule type	dna
Query Length	116

Descriptions

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Leptospira interrogans serovar Hardjo strain L53 chromosome 1, complete sequence	198	198	100%	3e-47	97.41%	CP043041.1
Leptospira interrogans isolate MBT46 LipL32 (lipL32) gene, partial cds	198	198	100%	3e-47	97.41%	MK328874.1
Leptospira interrogans isolate MBT85 LipL32 (lipL32) gene, partial cds	198	198	100%	3e-47	97.41%	MK328873.1
Leptospira interrogans isolate MBT84 LipL32 (lipL32) gene, partial cds	198	198	100%	3e-47	97.41%	MK328872.1
Leptospira interrogans isolate MBT74 LipL32 (lipL32) gene, partial cds	198	198	100%	3e-47	97.41%	MK328871.1

Fig 3: NCBI Blast Results of Human and Canine amplified products after sequencing

3.4 Results of the test samples

The samples were considered positive with threshold cycle (Ct/Cq) levels less than 40 (Fig.1). The average Ct value observed for positive samples from dogs with acute clinical signs was 29.67±0.5 cycles ranging from 10.86 to 36.20 cycles.

3.5 Hematological and serum biochemical parameters

There were abnormalities in hematological parameters studied, in 16 dogs which were showing acute clinical signs of leptospirosis. These dogs had significantly high total leucocyte count during febrile phase (22,000 to 26,000 cells/cumm). There were elevated levels in serum biochemical parameters-AST, ALT, ALKP, total bilirubin and creatinine, indicating renal and hepatic involvement. Most of the dogs presented with acute clinical signs were treated immediately for the cause with Antibiotic-Benzyl Penicillin (Penicillin G) @ 40,000IU/Kg bodyweight intravenously q 24 hrs. for 7 days and oral amoxicillin clavulanate @ 10 mg/kg TID q 8hrs, doxycycline@10mg/kg q 24 hrs. for 21 days. Out of sixteen dogs- two dogs presented with severe clinical signs of ascites and icterus and one cat succumbed to death within two days after identifying the etiological agent and initiating treatment, rest of the dogs and one cat survived. Dogs presented with severe clinical signs of leptospirosis had icterus and ascites, but cats were showing clinical signs of cystitis with blood in urine and pyrexia which was not initially suspected for leptospirosis. The clinical signs described previously viz; rise in temperature, congested mucus membrane, ascites and icterus can be suggestive of acute clinical signs of leptospirosis and the results of present study indicate that the taqman based qualitative real time PCR (qPCR) can be used as an accurate and confirmatory diagnostic method to detect the presence of pathogenic leptospires in EDTA blood samples. This can be used as a diagnostic tool not only for human or canine cases presented with acute clinical signs but also helpful to identify carrier status. The present study also gives an insight to the fact that feline cases presented with symptoms like blood in urine not responding to routine antibiotic therapy should also be ruled out for leptospirosis and RT PCR protocol standardized in present study can be very useful.

4. Discussion

Leptospirosis is a disease which is often diagnosed late due to its wide spectrum of symptoms ranging from a flu like syndrome to renal failure and it mimic the clinical presentations of many other diseases in canines (tick transmitted diseases) and human beings (dengue and malaria) [1]. Currently available serological diagnostics for humans and canines do not allow the confirmation of clinically suspected leptospirosis at the early acute phase of illness. One of the most important diagnostic tests, the microscopic agglutination test (MAT), which is often used as the gold standard, is based on serology and can only confirm the disease at a later acute phase because anti-leptospira antibodies generally become detectable only 5 to 7 days after onset of illness. Thus to enable starting treatment at the most effective time point, the availability of an accurate diagnostic test that is reliable in the early acute phase of the disease, is essential [2]. The PCR technique, which can detect the DNA of pathogenic leptospires present in the blood of the patient in the first 5 to 10 days, is a promising tool for early laboratory diagnosis of leptospirosis [2]. PCR of blood samples can rapidly confirm the diagnosis in the early phase of the disease (within the first 2 weeks of exposure) before antibody titers are at detectable levels [1]. Several conventional PCRs for the diagnosis of leptospirosis have been described, using a variety of target genes, including rrs [4], flab [5], ompL1 [6] and G1orG2 [7]. A number of real-time PCRs were introduced as a rapid and sensitive tool for leptospires detection, reducing the risk of false positive results by carry-over contamination. PCRs targeting the *lig A*, *B* genes [8], *rrs* gene [9], *gyrB* [10], gene the conserved hypothetical protein coding locus LA0322 in *L. interrogans* serovar *Lai* [11], and *lipL32* [12, 13], are claimed to be specific for pathogenic *Leptospira* and therefore appropriate for diagnostic purposes. Taqman chemistries targeting *lipL32* gene-which is a highly conserved sequence

of pathogenic leptospires, from whole blood samples, plasma and serum of both humans and canines had been attempted by several workers earlier [1]. Majority of RT PCR studies have indicated a primary role for *lipL32* gene which encodes for the *Leptospira* subsurface lipoprotein [14]. Because *lipL32* is believed to be a virulence factor that is only presented in pathogenic species, this provides for the selective detection of the pathogenic *Leptospira* and helps to increase the specificity of these methods [15, 16]. RT PCR targeting the *lipL32* gene was faster, more sensitive and more specific for determination of *Leptospira* DNA in clinical samples like blood and urine from human patients [2]. Higher sensitivity for the RT-PCR was documented [3] earlier which confirmed leptospira infection in 22 samples (from 21 patients), while traditional PCR revealed negative results. In the present study also taqman chemistry based RT PCR protocol standardized in our laboratory could rapidly identify *lipL32* gene in all samples tested- 16 dogs, 2 cats and 2 humans. Rats are the primary reservoir of leptospirosis, although farm animals and livestock, such as horses, pigs, dogs or cattle, and even wild animals can also be a reservoir for the bacteria. However, human-to-human transmission seems to occur occasionally. It is also an occupational hazard with potential risk of exposure among outdoors workers such as farmers, cleaners, veterinarians, agricultural workers. Moreover, there exists an increased chance of a recreational hazard to those who swims and wades in contaminated waters. The fact that clinically normal dogs can be chronic carriers of infection and may shed *Leptospira sp* to the environment is well documented [17]. RT PCR targeting *lipL32* gene to study urinary shedding of pathogenic leptospira has been documented earlier [18]. Out of 239 urine samples collected from clinically normal dogs from 13 districts of Haryana, none of the samples were found positive for pathogenic leptospira indicating low risk of transmission from dog's urine to human beings in that particular districts of Haryana. In a study conducted in Mumbai during 2006 to 2008 among dogs and rodents- using 100 blood and 18 urine samples, leptospira DNA was detected by RT PCR in 2 canine blood and 5 urine samples of rodents and high sero prevalence was noted among dog population proving possible role of dogs and rodents in transmission of leptospirosis to human beings [19]. In present study 46% of dogs under study which were undergoing treatment for multiple tick transmitted diseases were harbouring *lipL32* gene of pathogenic leptospires indicating environmental exposure to pathogenic leptospires. Since leptospirosis can mimic clinical presentations of many tick borne diseases, dogs under study were also responding to oral doxycycline therapy during treatment schedules, possibility of overlap of clinical signs and improper diagnosis of leptospirosis also could not be excluded. RT PCR standardized in present study can be a useful tool for confirmatory diagnosis in such cases. According to findings of [17] 500 dogs (only 4 had clinical leptospirosis others normal) were when evaluated serially irrespective of health status during a six-month period 8.2% were shedding pathogenic leptospires and such clinically normal dogs may pose a zoonotic risk to their owners. Hence in the present study risk assessment of transmission from RT PCR positive dogs to their owners and to other healthy dogs through contaminated urine must be the next important point of concern. Cats are exposed to leptospires regularly particularly by their habit of hunting infected rodents but clinical leptospirosis is rare in cats, infected cats shed pathogen through urine and can play a major role in epidemiology of leptospires [20]. In present study also two cats presented were not showing classical clinical signs like dogs, but had cystitis with blood in urine indicating possible involvement of kidneys and urinary system with shedding of leptospires in urine. In another study in Thailand [21] among 260 outdoor cats using RT PCR targeting *lipL32* gene in urine samples 0.8% were PCR positive indicating cats can be a potential source of infection even though with low prevalence

Results of present study also gives a clear cut evidence that the disease leptospirosis can occur in cats and they can play a possible role in transmission of pathogenic leptospires to surrounding environment.

To conclude RT PCR protocol standardized in present study based on taqman chemistry can be a very useful tool to diagnose pathogenic leptospires in human, canine and feline EDTA blood samples collected during febrile phase which can give a confirmatory diagnosis to practitioners at the early acute phase of illness itself and need not wait for sufficient antibody titres to develop for a serological diagnosis. Moreover, this study also emphasises that cats presented with symptoms of cystitis and fever not responding to routine antibiotic therapy also should be ruled out for leptospirosis and RT PCR protocol can be a very useful tool to arrive at a confirmatory diagnosis. This protocol also can be used to study the carrier status of pathogenic leptospires in those animals not exhibiting classic clinical signs or animals suspected for other diseases and undergoing treatment. To authors knowledge this is the first report of qualitative (qPCR) based RT PCR diagnostic protocol based on taqman chemistry for detection of pathogenic leptospires in human, canine and feline blood samples in Thrissur district, Kerala State.

5. Acknowledgments

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6. Competing interests

The authors declare that they have no competing interests.

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