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Research Article

Detection of Pathogenic Leptospiraserovar in Malaysian Wild Rats by Real Time PCR based on LFB1 Gene -

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Abstract

Conventional laboratory diagnosis of Leptospirosis is laborious and time consuming. Real time PCR has been reported as cost effective for rapid diagnosis of leptospirosis. In this study, the Real Time PCR method based on LFB1 genes were used to detectpathogenic Leptospira spp in wild rats in selected location in Kuala Lumpur Malaysia (Chow Kit and Dato Keramat market). 140 rats were captured from that area, and samples from kidneys were collected for culture.All the isolates which were obtained were cultured in EMJH supplemented with 3 % rabbit serum. 4 reference genes which are Leptospiracanicola, Leptospirajavanica, Leptospirabataviae and Leptospirahardjo were used to monitor the detection of Leptospira spp. 25 samples were used including one negative sample to detect Leptospirasp. By using Agilent Brilliant III SYBR Master. Mix that consist a novel mutant Taq DNA polymerase which enhance higher nucleotide incorporation and a novel faster-activating hot start method which helps to minimize formation of primer-dimers and increase the specificity of target detection. The primers used were LFB1-F' and LFB1-R' for target detection. This gene has been reported to be responsible for the pathogenicity of Leptospira interrogans. All 4 reference gene were successfully amplified and all the 4 reference genes showed the same melting temperature which is 81°C. All amplified samples categorized into two genospecies as two distinct melting curve observed which was 81°C and 83.5°C respectively. In the real time PCR assay a positive reaction was detected by the accumulation of a fluorescent signal calculated in Ct (cycle threshold).All the control pathogenic leptospira produce Ct values ranging from 30.09 to 32.10. Twenty leptospira isolates from the wild rats gave Ct value ranging from 14.27 to 20.22. This study shows that Real Time PCR using the above primer set could be used to identify pathogenic leptospira from local wildrat's samples.

INTRODUCTION

Leptospira is the etiologic agent of leptospirosis, a bacterial zoonosis with worldwide distribution. Leptospirosis is caused by *Leptospira*sp, a spirochete aerobic bacterium, gram-negative, with spiral morphology [1]. It is an important global disease with public and animal health implications [2]. The number of patients in Malaysia infected with leptospirahas increased lately. An outbreak of leptospiral infection was reported among athletes participating in the Eco-Challenge-Sabah 2000 held in Malaysian Borneo. The infection was reported to be associated with water-related activities. Investigation of large outbreaks would be greatly enhanced by the availability of rapid and sensitive diagnostic assays which can confirm the diagnosis early in the clinical illness [3]. There are over 230 known serovars in the genus *Leptospira*. The disease is maintained in nature by chronic renal infection of carrier mammals, which excrete the organism in their urine [4]. A study conducted in 2009 in Malaysia indicated that *Leptospira*serovar were prevalent in the Malaysian rat population and could be a source of infection to humans. Diagnosis of leptospirosis is usually accomplished retrospectively by serology, because culture requires both special media and incubation for several weeks [5]. Serologic diagnosis by microscopic agglutination test invariably requires testing of acute and convalescent sera, since agglutinating antibodies soften are not detectable during the acute illness. IgM antibodies become detectable 5 -7 days after the onset of symptoms, and the use of IgM-ELISA assays for presumptive diagnosis has been evaluated in numerous populations [6]. Since leptospira are difficult to culture, several PCR methods have been used to facilitate early diagnosis [7]. A number of PCR assays for leptospiral DNA have been described, but only two have been evaluated in clinical studies used extensively for diagnosis [8]. PCR assays which could detect all pathogenic and non-pathogenic leptospires in clinical samples were also described [9]. More recently, a real-time PCR was developed using Taq Man chemistry (which targeted an 87bp section of the16S rRNA gene of *Leptospira*sp [10]. We describe here detection of **pathogenic leptospiraserovarin Malaysian wild rats by RealTime PCR based on LFB1 gene** using SYBER Green kit**.**

MATERIALS AND METHODS

Origin of leptospira isolate samples

The choice of the study area is based on habitat suitability rats for food, livestock breeds and the possibility of spreading the disease. Catching rats from two selected areas in Kuala Lumpur , Datuk Keramat (03 ° 09'57.00 " N , 101 ° 43'52.00 " E) and Chow Kit (03 ° 09'53.75 " N , 101 ° 41'56.84 " E) Malaysia conducted in collaboration with Kuala Lumpur City Hall (DBKL), Datuk Keramat selected because of features like roadside stalls selling cooked food is also member food for rats. Besides, poor garbage management also attract rats, stray dogs and cats for breeding and food sources. Chow Kit is the largest fresh food market in Kuala Lumpur. Provide a variety of raw foods, including fruits, vegetables, seafood, and meat to the public. There is a lot of waste dumped into the steel container 30. Excessive garbage falls to the ground and attract rats to get as a food source. The arrest was carried out by using a metal cage measuring 18 x 12 x 28 cm with bait food like coconut, sweet potatoes or bread. Cage placed in areas that are consistent with the evening and retrieval is done in the early morning before the area was filled with people.

Culturing samples to EMJH

As many as two drops of blood were collected using cardiac puncture in the vein or dripped into a tube containing 5 ml of media Leptospira sp. EMJH containing 200 ug / ml 5 - fluorouracil and two drops of urine taken from mice using direct puncture dripped on a 5 ml tube containing media Leptospira sp. EMJH 200 ug / ml 5 - fluorouracil. Samples were incubated for 2 months at 28 °C - 30 ° C in the dark.

Samples were observed using a dark field microscope on the first day (one day after the culturing) until the third day, followed by the first week until the eighth week. A total of 1.5 ml of the first culturing containing Leptospira sp. transferred into the new media to get a pure culture. Contaminated culture filtered using a syringe filter size of 0.45 nm.Leptospira isolates cultivated in EMJH medium supplemented with 0.21 % rabbit serum. The culture were harvested in late log phase after incubating on ice. The tube was cooled on ice for 10 minutes, vortexed and spun at 15g for 10 minutes. After removal of the supernatant, an equal volume of PBS was added to the pellet and the content centrifuged for 10 minutes at 15g. The bacteria were transferred to an appropriate container for further work.

Genomic DNA extraction and PCR

The culture were washed 3 times, centrifuged at 3000 rpm (10 minutes each time) and processed immediately for DNA extraction. The DNA was extracted using Qiagen DNA Extraction Kit (USA). All the six local isolates were subjected to PCR with specific primers

(Table 1). The PCR was performed with 2.5 units of the Taq DNA polymerase (Fermentas AB, Lithuania) in a reaction mixture (100µl) containing dNTPs (200µM) and 2.5mM $MgCl_2$, subjected to 10 cycles of 1 minute at 95°C (denaturation), 30 seconds at 58°C (annealing) and 1 minute at 72°C (extension). The amplification product was purified using High Pure PCR Product Purification Kit (Boehringer Mannheim, Germany).

Real Time PCR

Real time thermal cycler used were Bio-Rad CFX for the detection SYBR Green (SYBR) in the samples. LFB1-F (5'-CATTCATGTTTCGAATCAT-TTCAAA-3') and primer LFB1-R (5' Primer GGCCCAAGTTCCTTCTAAAAG-3') chosen in the locus LA 0322 of L.interrogansspp sequence were used to amplify a 331-bp product from pathogenic *Leptospira* spp.

RESULTS AND DISCUSSION

A rapid, simple and accurate method is needed for diagnosis of leptospirosis. Direct demonstration of *Leptospira* clinical samples such as blood, urine, CSF performed by bacterial culture takes too long time and is insensitive (Ellinghausen Serum antibody detection, thus, serves as an indirect alternative means of leptospirosis diagnosis. MAT is one of the commonly used antibody detection assays [11]. MAT is insensitive during the early phase of infection and requires a large battery of living *Leptospira spp*. of various sero groups and serovars which are laborious and costly [12]. Other simpler antibody detection methods have been developed, such as indirect immunoflorescent assay, IgM ELISA and IgM dipstick. Sensitivity of these assays, however, is still limited by the *Leptospira*spp. Used for preparing the antigens. False negative results may occur if the infecting *Leptospira spp.* does not match the *Leptospira*spp. Used as antigen in the assays. Alternatively, false positive results may be obtained by antibodies in serum of patient who have been infected by previously unrecognized *Leptospira* infection or exposure to antigenically related organisms especially in the leptospirosis endemic areas [13]. In this study, Real time PCR techniques was applied to detect the presence of pathogenic leptospiral DNA in isolates samples of wild rats in selected area in Chow Kit and Dato Keramat Market in Kuala Lumpur Malaysia.

Four pathogenic isolates were used as positive control. The cultured cultures were *LeptospiraCanicola, LeptospiraBataviae, LeptospiraHardjo* and *Leptospirajavanica.*The isolates were obtained from Unit Bacteriology Institute Medical Research. (Figure 1) (Figure 2) showedamplication plot of 4 reference genes and amplification of all samples respectively. In the real time PCR assay a positive reaction was detected by the accumulation of a fluorescent signal calculated in Ct (cycle threshold): All the control pathogenic leptospiras produce Ct values ranging from 30.09 to 32.10 (Table 1). Twenty leptospira isolates tested from the wild rats gave Ct value ranging from 14.27 to 20.22 (Table 2). In this study, real time PCR technique was applied to detect the pathogenicity of20 local isolates from wild rats. The local isolates are from wild rats selected market from Chow Kit (S1, S2, S3, S4, S5, S6, S7, S8, S9) and wild rats selected market from DatoKeramat (S10, S11, S12, S13, S14, S15, S16, S17, S18, S19 and S20) in Malaysia. All the isolates were cultured in EMJH supplemented with 0.21 % rabbit serum. Real Time PCR with SYBER Green kit were used to amplify the extracted DNA using the set of primers known to amplify the locus LA0322 of Leptospira. This gene is reported to be responsible for the pathogenicity of *Leptospirainterrogans*.

Table 1: Ct Value Real Time PCR amplified DNA using **LFB1 gene** from reference pathogenic strain *leptospira* sp.

No.	SAMPLE	Ct VALUE
1	*Leptospira Canicola*	30.27
2	*Leptospira Bataviae*	31.06
3	*Leptospira Hardjo*	30.99
4	*Leptospira Javanica*	31.97

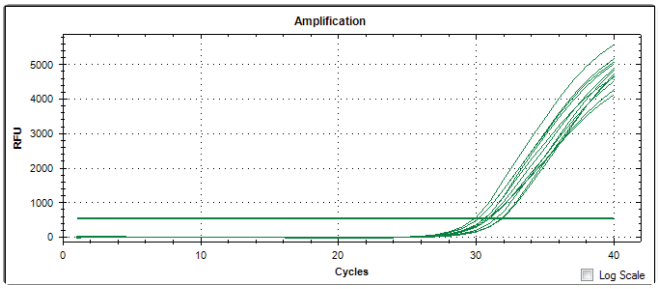

Figure 1: Amplication plot of 4 reference genes samples for real time PCR for LFB1 gene

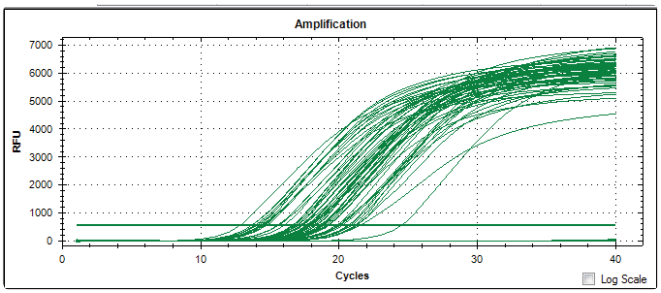

Figure 2: Amplication plot of all samples for real time PCR for LFB1 gene.

Table 2: Ct Value Real Time PCR amplified DNA using **LFB1 gene** from various isolates from rats (Chow Kit Market).

No	SAMPLE	CT VALUE	ORIGIN OF SAMPLES
1	S1	19.03	Chow Kit Market
2	S2	17.68	Chow Kit Market
3	S3	17.63	Chow Kit Market
4	S4	21.22	Chow Kit Market
5	S5	20.90	Chow Kit Market
6	S6	18.49	Chow Kit Market
7	S7	17.52	Chow Kit Market
8	S8	20.22	Chow Kit Market
9	S9	14.27	Chow Kit Market
	Blank (negative)		

Melting curve analysis showed specific amplifications which allow identification of control pathogenic leptospira (Figure 3). All of the control pathogenic leptospira gave similar melting temperature of 81^{0}C. Meanwhile local isolates gave melting temperature either similar to control pathogenic leptospira (Table 3) or a higher melting temperature of 83.5^{0}C (Table 4). These enable accurate grouping between the possible pathogenic and non- pathogenic leptospira in local isolates**.** Therefore Real Time PCR with SYBR Green as

described has potential as a diagnostic tool in identifying pathogenic leptospira from local wild rats.

Figure 3 and 4 showed melting curved and melting peak of all samples tested. For all amplified samples seems to be categorize into two genospecies as two distinct melting curve is observed. One is at 81°C meanwhile the other group have melting temperature of 83.5°C. Out of the 20 positive samples, 6 local isolates melting temperature are 81°C and 14 local isolates have melting temperature 83.5°C. The leptospira isolates from wild rodent are S7, S9, S12, S17, S10 and S15 exhibited a melting curved compatiblewith all the referecestrain [*LeptospiraCanicola, LeptospiraJavanica, LeptospiraBatavie,* and *LeptospiraHardjo*]. Therefore, there was a possibility that those isolates were also pathogenic. These data shows potential of LFB1 real time assay for the detection of pathogenic leptospira from environment. Further research should be done to detect *leptospira*in animal and human samples using this method

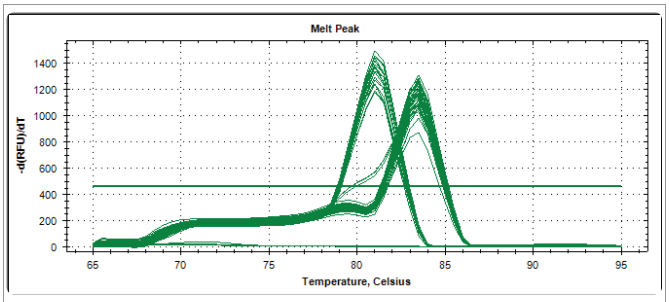

Figure 3: Melting curve of all samples.

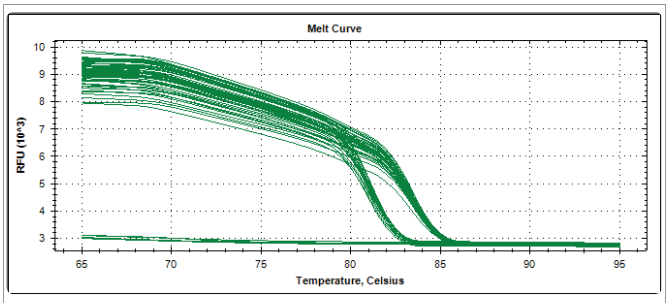

Figure 4: Melting peak of all samples.

Table 3: Ct Value Real Time PCR amplified DNA using **LFB1 gene** from various isolates from rats (Dato Keramat Market).

No	Samples	Ct Value	Origin of samples
1	S10	18.61	Dato Keramat Market
2	S11	21.13	Dato Keramat Market
3	S12	14.12	Dato Keramat Market
4	S13	17.03	Dato Keramat Market
5	S14	18.54	Dato Keramat Market
6	S15	18.04	Dato Keramat Market
7	S16	14.72	Dato Keramat Market
8	S17	15.76	Dato Keramat Market
9	S18	18.45	Dato Keramat Market
10	S19	14.19	Dato Keramat Market
11	S20	19.30	Dato Keramat Market
	Blank(negative Control)		

Table 4: Various isolates samples from reference (pathogenic strain) and wild rats with melting temperature: 81° C.

Sample	Melting temperature
Leptospira Bataviae	81° C
Leptospira Canicola	81° C
Leptospira Hardjo	81° C
Leptospira Javanica	81° C
S7	81° C
S9	81° C
S12	81° C
S17	81° C
S10	81° C
S15	81° C

CONCLUSION

Pathogenic leptospires from environment can be detected using a real time PCR assay based on SYBR Green technology. This method is rapid and simple and could be apply in human and animal too.

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