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Journal Articles

DETERMINATION OF PREDOMINATE *LEPTOSPIRA* SEROVARS CIRCULATING AMONG RAT POPULATIONS IN KUALA LUMPUR

G.AL Kattan, A.R. Bahaman*, S. Khairani- Bejo, A.R. Mutalib and Z. Zakaria

Faculty of Veterinary Medicine, Universiti Putra Malaysia, UPM Serdang, Selangor, Malaysia.

SUMMARY

Leptospirosis is re-emerging in Malaysia and many other tropical countries. Its infection of human and animal's worldwide. The study was carried out to identify predominate *Leptospira* serovars within rat populations in Kuala Lumpur (KL) by microscopic agglutination test (MAT). As well to investigate the frequency of infection by polymerase chain reaction assays (PCR). The isolated cultures that obtained from culturing kidney tissue of 112 trapped rats from four sites in KL, were identified by MAT using panel of 16 standard hyper immune anti-sera. Besides, identification of *Leptospira* strains by PCR amplification with G1/G2 and ompL1 *Borgpetersenii* genes. Results from typing by MAT revealed that 8/57 (7.1%) of cultured isolates reacted against; hyper-immune antisera of serogroup Javanica and 13/57 (22.8%) against serogroup Bataviae. Whereas, the rest of isolates recorded across reactivity 1/20 against serogroups; Icterohaemorrhagiae 2/57, Canicola 1/57, Australis 2/57. From PCR-G1/G2; 50/112 (50.9%) cultures were positive included (40/57 cultures positive to isolation and 10/55 cultures negative to isolation). While, from PCR-ompL1 *Borgpetersenii* gene; 21/112 (18.7%) cultures positive included (17/57 cultures positive to isolation and 4/55 cultures were negative to isolation). It is obvious from this study that serogroups Javanica and Bataviae were the predominant among rat populations in KL. Also it revealed the high frequency of pathogenic strains among rat populations and their potential risk of humans and animals contracting infection.

Keywords: Bataviae, Javanica, Leptospirosis, MAT, PCR.

INTRODUCTION

Leptospirosis is a communicable disease of animals and humans caused by infection with *Leptospira* spirochaets (Pappas *et al*., 2008). Leptospirosis has been recorded as endemic in Malaysia since four decades. Several outbreaks have occurred recently e.g. the Lubuk Yu outbreak (Sapian *et al*., 2012). Humans may contract the infection through direct contact with infected animals or indirect contact with urine contaminated materials; soil and water (Blackwell, 2014). Therefore, the goal of this study was to identify the serovars circulating among rat populations in Kuala Lumpur (KL) by microscopic agglutination test (MAT) and to investigate the frequency of infected serovars among the rat population in the urban.

MATERIAL AND METHODS

Sampling locations

Four sites were chosen in KL based on certain measures that Malaysia is a tropical region and the locations were public markets; Pudu market, Raja Bot market, Selayang market and Sentul market. Trapping methods were in accordance to authorisation; Project no: UPM/IACUC/AUP-R078/2016. Rats were trapped using the metal wired traps the night before a day of sampling.

**Corresponding author: Prof. Dr. Abdul Rani Bahaman (A.R. Bahaman); E-mail: ranibahaman3@gmail.com.*
First author: Dr. Ghada AL. Kattan (G.AL. Kattan);
Email: gaalawsi2013@yahoo.com.

Animals

Rats (n=112) were trapped from four selected sites in KL by the *Dewan Bandaraya Kuala Lumpur* (DBKL). Trapped rats were euthanised by inhalation of 60% carbon dioxide for 5 min in a compressed small cylinder set fully enclosed chamber). The rats were dissected and the kidneys were collected aseptically.

Isolation of Leptospira spp. from cultured rat kidneys

All kidneys were cultured after washing by sterile distilled water in sterile cabinet. Culturing and isolation of leptospires were done based on the methods used by Faine (1999).

Microscopic agglutination test (MAT)

A well grown cultured isolates of $4x10^7$ cell/ml in Ellinghausen, McCullough, Johnson, and Harris (EMJH) liquid medium were prepared to carry out the MAT. The prepared cultures were tested against 16 rabbit hyper immune antisera, these sera exemplifies of 16 essential serologic groups of *Leptospira* species in Malaysia (OIE, 2014). Microscopic agglutination test (MAT) was performed according to (OIE, 2014) standard. A set of 16 reference Rabbit hyper immune antisera (Hebdomadis [Hebdomadis], Hardjobovis [Hardjobovis], Bataviae [Swart], Harjdo [Hardjoprajitno], Pyrogenes [Salinem], Cynopteri [3522c], Tarrassovi [Perpelitisin], Mini [Sari], Ballum [Mus127], Autumnalis [Akiyami], Grippotyphosa [Moskvav], Celledoni [Celledoni], Javanica [Veldratbataviae46], Australis [Ballico], Icterohaemorrhagiae [RGA], and Pomona [Pomona])

demonstrated as the main serovars in Malaysia were obtained from the Queensland Laboratories, Australia, and used to determine the serogroups for each of the positive isolates. A positive result for MAT was obtained when there was a 50% agglutination following reaction of the isolates with the hyper-immune anti-sera (*Leptospira* culture diluted 1:2 in the sterile phosphate buffer only). Sterile saline buffer of 50 µl were dispensed in all wells and 40 µl of sterile PBS added to the second raw only. Then, the standard sera of 10 µl were added to wells of second raw only. To make double dilution 50 µl of sera were transferred to all wells starting from second raw. Finally, 50 µl of bacterial culture of no more than seven days old were dispensed in all wells, starting from first raw. The plates were covered and incubated at 37°C for two hours. A drop from each well transferred to a glass slide and observed under dark felid microscopy for reaction of agglutination. The isolated sample was regarded to be positive when approximately 50% or more of the examined leptospires were agglutinated.

Isolation of genomic DNA

Cultures of kidneys tissue (n=112) were prepared in fresh liquid EMJH. The isolation of DNA was performed using Wizard Genomic DNA purification kit (Qiagen 2010, USA), following the manufacturer's instructions.

PCR assay

The extracted DNA was amplified by PCR primers G1/G2 and *OmpL1 borgpetresinii* gene designed for pathogenic *Leptospira* isolates (Gavekamp *et al*., 1993; Raven *et al*., 2006) in 25µl reaction volumes following instructions of (Qiagen 2010, USA) and carried out in thermal cycler (Eppendorf). The protocol of the PCR-G1/G2 reaction entails 30 cycles of denaturation at 94^{O}C for 60 sec after the initial incubation at 94^{O}C for 5 min, annealing at 55^{O}C for 60 sec then, elongation at 72^{O}C for 2 min, last elongation at 72^{O}C for 10 min. The protocol of the PCR- *OmpL1 borgpetresinii:* the initial incubation at 94^{O}C for 5 min, followed by 30 cycles of denaturation at 94^{O}C for 30 sec, the annealing at 58^{O}C for 30 sec then, extension at 72^{O}C for 30 sec and final elongation at 72^{O}C for 7 min.

Gel electrophoresis

Approximately 5 µl of PCR product were mixed with 2 µl of blue (6×) gel loading dye. Then, 7 µl of the mixture were loaded into the gel wells. The electrophoresis was run on 1.5% agarose gel in 1.5% Tris-Borate-buffer (TBE), at 100 Voltage for 60 min. The gel was stained by Ethidium bromide (10 µg/ml) for 5 min and distained by distilled water for 15 min then, PCR products seen by Alpha imager.

RESULT

From isolation: A total of 57/112 cultures were positive to presence of leptospires when tested by dark field microscopy (DFM).

From result of MAT: It was seen that; 13/57 (22.8%) isolates were reacted against hyper-immune antisera of serogroup Bataviae with titer ranging from 1/80 to 1/1280 and 8 (7.1%) isolates were reacted against Javanica serogroup with titer ranging from 1/40 to 1/80.Whereas, the rest of isolates were recorded with cross reactivity 1/20:2/57 against Icterohaemorrhagiae, Australis and 1/57 against Canicola (Table 1). Based on PCR assay: 50/112 (44.6%) were amplified DNA by PCR-G1/G2 primers and successfully yielded a product of expecting size (285bp). The 50/112 amplified samples were included 40/57 (35.7%) samples from cultures positive to isolation and 10/55 (8.9%) samples from cultures negative to isolation. Whereas, amplification by PCR-*OmpL1 borgpetresinii* gene gained 21/112 (18.7%) positive products of expecting size (400bp) included: 17/57 (29.8%) cultures were positive to isolation and 4/55 (7.27%) culture were negative to isolation.

DISCUSSION

In the present study, *Leptospira* strains obtained from cultured rat kidney were typing by MAT and identified by PCR. Rats have been recognised as the most common reservoirs of *Leptospira* (Kosoy, 2015). Base on MAT in this study, the predominated serogroups were Bataviae and Javanica with titer >1:400. This result agreed with result of last study though they recoded both serogroups, in lower per cent (Benacer *et al*., 2013). MAT is a gold standard method for *Leptospira spp*. typing but due to the need of long-term maintenance of large range of reference strains and the standard hyper immune antisera, MAT has been applied only in a limited number of reference laboratories. Besides, pathogenic *Leptospira spp*. including more than 230 serovars, the majority of them has no corresponding specific antisera and cannot be identified by MAT (Ahmed *et al*., 2006; Thaipadungpanit *et al*., 2007).

Table 1: Frequency titer of the recovered *Leptospira* isolates serogroup

Serogroup Antisera	Titer								Total
	20	40	80	160	320	640	1280	2560	
Javanica	0	6	2	-	-	-	-	-	8
Bataviae	-	-	1	3	5	3	1	-	13
Ictero	2	-	-	-	-	-	-	-	2
Canicola	1	-	-	-	-	-	-	-	1
Autralis	2	-	-	-	-	-	-	-	2
Total	5	6	3	3	5	3	1	0	26

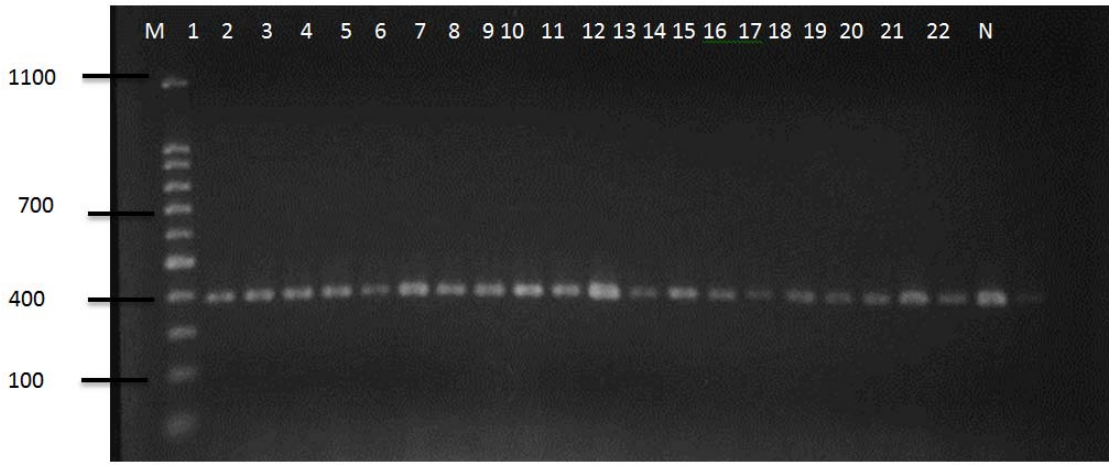

Figure 1. PCR Amplification of 400 bp *omp L1 Borgpetersinii* gene: Lane M= Molecular DNA marker 100 bp, Lane 1-20 positive control Javanica, Lane 23 negative control, Lane 2-22 positive samples

However, combining MAT with molecular assay generated confirm results. Based on PCR; G1/G2 primers detects all pathogenic species except *L. Krishrerii.* Whereas, *ompL1 Borgpetersenii* detects *L. Borgpetersenii spp*. only. By using PCR as sensitive tool for detection of leptospires in small number, the number of detected isolates was raised from 26 isolates that detected by MAT to 40/57 isolates that detected by PCR. Besides, PCR-G1/G2 and *ompL1* gene detected 10 and 4/55 positive samples respectively cultures were negative for isolation. Results of this study agreed with results of Boonsilp (2011). Detection of leptospires in cultures negative to isolation could be speculated that some of strains are fastidious and require complicated nutrition's hence, fail to grow in visible number. In this study, PCR was found to be highly sensitive and specific in detecting the real number of infected specimens and to identify the pathogenic isolates. This technique overcomes the problems of complex isolation and serologic identification of *Leptospira spp.* involved in infection. Therefore, PCR yielded a real data on the epidemiology of leptospirosis and their maintenance host in the country. Applying the PCR assay for directly screening of cultured isolates is more sensitive. Hence, PCR assay has proven to be sensitive to detect a very small number of leptospires in cultures that detected negative to isolation. From the results, it is seen that poor sanitation and hygiene in urban slums generates an ideal environment for rat proliferation. Dweller and workers there should be educated of how to keep their environment clean especially how to discard garbage in a correct or proper manner.

CONCLUSION

From this study, *L. interogans serogroups* Bataviae and *L. Borgpettersenii* serogroup Javanica were predominated among rat population in KL. In addition, combining classical methods with molecular assays has advantage over using classical methods only. So, further studies are required to determine the pathogenicity of circulating serovars among rat population and their role in human leptospirosis in the country. Continues monitoring for hygiene and set a scheme to control rats could reduce the threat of leptospirosis.

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CONFLICT OF INTEREST

None of the authors have any potential conflicts of interest to declare.

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