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Detection of pathogenic Leptospira from selected environment in Kelantan and Terengganu, Malaysia

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Abstract. Leptospirosis is recognized as one of the important zoonotic diseases in the world including Malaysia. A total of 145 soil and water samples were collected from selected National Service Training Centres (NSTC) in Kelantan and Terengganu. The samples were inoculated into modified semisolid Ellinghausen McCullough Johnson Harris (EMJH) medium, incubated at room temperature for 1 month and examined under the dark-field microscope. Positive growth of the leptospiral isolates were then confirmed with 8-Azaguanine Test, Polymerase Chain Reaction (PCR) assay and Microscopic Agglutination Test (MAT). Fifteen cultures (10.34%) exhibited positive growths which were seen under dark field microscope whilst only 20% (3/15) were confirmed as pathogenic species. based on 8-Azaguanine Test and PCR. Serological identification of the isolates with MAT showed that *hebdomadis* was the dominant serovar in Terengganu. Pathogenic leptospires can be detected in Malaysian environment and this has the potential to cause an outbreak. Therefore, precautionary steps against leptospirosis should be taken by camp authorities to ensure the safety of trainees.

INTRODUCTION

Leptospirosis outbreaks have recently been reported in some developing countries including Malaysia. *Leptospira interrogans* is the main causal organism of this infectious disease and rats are believed to be the major natural reservoir for this bacteria (Bahaman *et al.* 1987). Leptospires can survive for long period of time in the environment under favorable conditions. Moisture, pH values of soil and surrounding temperature will influence the survivability of the pathogenic leptospires which can lead to high incidence of leptospirosis (Gordon, 1977). There are two modes of transmission for leptospirosis: direct or indirect contact. Interactions between the maintenance host, contaminated environment and susceptible animals play a role in the transmission of leptospirosis.

Currently, 11 species of leptospires have been identified worldwide (Faine *et al.*, 1999) and 37 serovars have been isolated from animals and humans in Malaysia (Bahaman & Ibrahim, 1988). Besides that, Alexander *et al.* (1975) had successfully isolated 29 serovars from natural waters and wet soils. The isolation of pathogenic leptospires from environment in bacterial media is difficult. This is because free living saprophytic leptospires are able to grow rapidly in culture and hinder pathogenic leptospires from being isolated (Wilson & Fujioka, 1995). DNA based techniques, such as Polymerase Chain Reaction (PCR) and Bacterial Restriction Endonuclease DNA Analysis (BRENDA), and also protein based techniques of polyacrylamide gel electrophoresis and immunoblot are now fast developing methods which are more

dependable and able to classify leptospires isolates rapidly.

There is no recent isolation of leptospires from waters and soils in Malaysia after the work of Khairani-Bejo in 2001. She studied the presence of serovar *hardjo* in waters and soils from selected cattle farms in Malaysia and reported that all leptospiral isolates obtained were *L. biflexa*. This present study was to detect the presence of pathogenic leptospires in the environment after the outbreaks of leptospirosis affecting the youth trainees reported recently. The ability for pathogenic leptospires to survive for certain period of time may possibly lead to an outbreak of the disease in that area.

MATERIALS AND METHODS

Sample collection

A total of 145 water and soil samples were collected from 2 selected camps in the east coast peninsular Malaysia. Seventy four samples were collected from National Service Training Centre (NSTC) in Kelantan while another 71 samples were collected from Terengganu. The sample collection was done in April 2009. Water samples were collected from various places in both camps such as ponds, streams, drains and puddles. The temperature and pH of the water samples were recorded. Water samples were passed through sterile 0.22-µm pore size membrane filter and 5 to 10 drops was inoculated into the modified semisolid EMJH medium (Johnson & Harris, 1967). Soil samples were placed in a sterile 50 ml falcon tube and soaked in *Leptospire*-free distilled water. Then, it was mixed by vigorous shaking. The suspension was allowed to settle for 5 to 7 min before being filtered using 0.22-µm membranes. Five to 10 drops were inoculated into the same type of culture media. This medium was incorporated with 5-fluorouracil (5-FU) at a concentration of 100 µg /ml to minimize bacterial contamination. All enrichment cultures were incubated aerobically at room temperature for 30 days and examined for the presence of *Leptospira* by dark-field microscope. Leptospires have a hook-like end, very thin and motile. If the bacteria were not detectable after 30 days of incubation, the sample was considered to be negative. Presence of at least four *Leptospira* present in a microscopic field was considered as a positive result (Issazadeh *et al.*, 2009). The positive samples were separated and subcultured in modified EMJH liquid media which were later used for further test.

8-Azaguanine Test

The pathogenic leptospires could be differentiated from saprophytic leptospires by 8-Azaguanine Inhibition Test. Johnson & Rogers (1964) protocol had been followed during this experiment but with some changes. The leptospiral isolates and together with reference controls for pathogenic (serovar *icterohaemorrhagiae*) and saprophytic (*L. patoc*) were maintained in liquid media containing 225 µg/ml concentration of 8-Azaguanine. The cultures were incubated at 30°C and observed under dark-field microscope for growth rate by comparing them to the controls which made up of the same media but without 8-azaguanine. Pathogenic leptospires would not exhibit growth in 8-azaguanine media compared to saprophytic leptospires which are resistant to that compound.

Polymerase Chain Reaction

DNA extraction was performed on leptospira isolates by using the Wizard™ Genomic DNA purification Kit (PROMEGA). Seven to ten day-old cultures were used in this experiment. G1/G2 primers were used (Gravekamp *et al.*, 1993). Amplification of the DNA was conducted in a total volume of 25 µl. The reaction mixture consisted of 1X PCR buffer, 3.0mM of $MgCl_2$, 250µM of dNTP's premixed, 1µM of each primers and 0.5 unit of *Taq* Polymerase DNA. There were 3 steps that consisted of denaturation at 94°C for 1.5 minutes, annealing at 55°C for 1 minute and extension at 72°C for 2

minutes. The steps were repeated for 30 cycles with addition of 5 minutes before cycles and 1 minute after the last cycles for final extension of the primers. The PCR product together with loading buffer was subjected to electrophoresis on a 1.0% agarose gel in 1X TBE buffer at 65 V for 2 hours. The gel was pre-stained with ethidium bromide and photographed under Bio-Rad Gel Doc.

Microscopic Agglutination Test (MAT)
The procedure for MAT was done using the method of Cole *et al.* (1973) but tested with reference hyperimmune sera. A set of 17 hyperimmune rabbit antiserum were purchased from the Queensland Health Clinical and Statewide Services, Australia. The leptospiral isolates were cultured in liquid medium until 10^8 organisms per ml density. The agglutination between isolate and hyperimmune serum which gave the highest titer was considered to indicate the infective serovar. The battery of antisera which were used in this study is shown in Table 1.

Table 1. Hyperimmune Rabbit Antisera that were used in serotyping of the leptospiral isolates

No.	Serovar	Strain
1.	Australis	Ballico
2.	Autumnalis	Akiyami A
3.	Ballum	Mus 127
4.	Bataviae	Swart
5.	Canicola	Hond Utrecht IV
6.	Celledoni	Celledoni
7.	Cypnoteri	3522C
8.	Grippotyphosa	Moskva V
9.	Mini	Sari
10.	Hebdomadis	Hebdomadis
11.	Icterohaemorrhagiae	RGA
12.	Javanica	Veldrat Bat 46
13.	Pomona	Pomona
14.	Pyrogenes	Salinem
15.	Hardjo	Hardjoprajitno
16.	Hardjobovis	Hardjobovis
17.	Tarassovi	Perepelitsin

RESULTS

The overall results for detection of pathogenic leptospiral isolates from Kelantan and Terengganu camps are summarized in Table 2. Observation of positive growth of leptospires under dark field microscope is a preliminary step to identify leptospires. Leptospires have a hook-like end, very thin and motile. Through this observation, it showed that 10.42% (15 out of the 144 samples) were positive. It was seen that ten isolates out of 74 samples (13.51%) were seen in Kelantan while 5/71 isolates (7.14%) were from Terengganu. Interestingly, out of 117 water samples, only 12 (10.26%) of them and 3/28 (10.71%) soil samples exhibit positive leptospiral isolates. Differentiation of leptospires by 8-Azaguanine inhibition test indicated 8/15 (53.33%) isolates were resistant to the compound which was considered as saprophytes. For Terengganu isolates, only 3/5 isolates (60%) were not resistant to 8-azaguanine compound compared to Kelantan isolates which showed 4/10 isolates (40%).

Table 2. Identification of the leptospiral isolates by inhibition of 8-Azaguanine, PCR and MAT

Isolates	8-Azaguanine	PCR	MAT
K 2	–	–	–
K 4	–	–	–
K 8	–	+	–
K 9	+	+	–
K 32	+	+	–
K 33	+	+	–
K 34	+	+	–
K 36	+	+	–
K 37	+	+	–
K 43	–	–	–
T 14	–	–	–
T 31	+	–	–
T 108	+	+	1: 100 (hebdomadis)
T 110	–	+	1: 100 (hebdomadis)
T 112	–	+	–
Total Positive (%)	8/15 (53.33%)	10/15 (66.67%)	2/15 (13.33%)

+ : growth/ have band
– : no growth/ no band
Code K: Kelantan isolates
Code T: Terengganu isolates

Subsequently, Polymerase Chain Reaction (PCR) assay was done for confirmation of pathogenic leptospires by using G1/G2 primers. The PCR results demonstrated that 66.67% (10/15) were confirmed to be pathogenic through formation of 285 base pairs product in 1% agarose gel (Figures 1 and 2). Both camps showed remarkable result which for Kelantan isolates, 70% (7/10) produced positive PCR product while 60% (3/5) for Terengganu isolates. Microscopic Agglutination Test (MAT) was basically done to screen the leptospiral isolates so that their serovar can be roughly identified. All 17 set of reference hyperimmune sera representing the important serovars in Malaysia. Upon testing with the 17 sera, the findings showed that 13.33% (2/15) were identified as serovar *hebdomadis* and were seen among Terengganu isolates only. Basically, it can be considered as most prevalent serovar in this study.

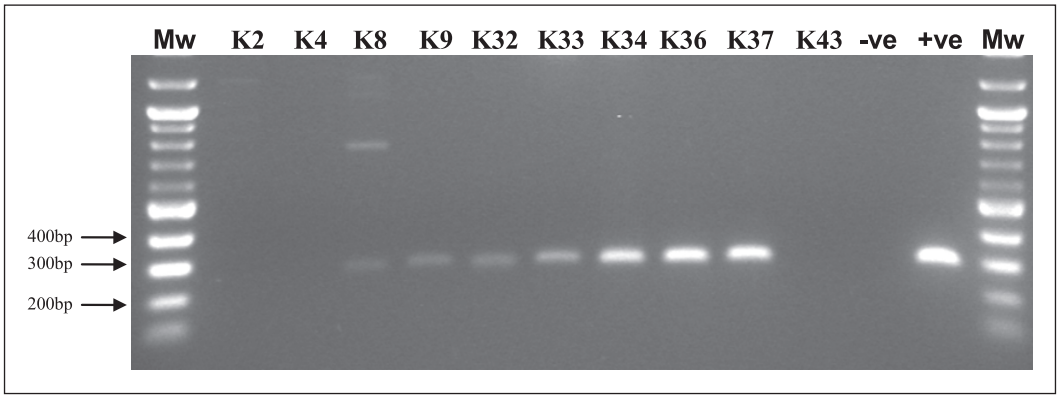

Figure 1. Confirmation of pathogenic leptospires on Kelantan isolates by PCR assay using G1/G2 primers
Mw: Molecular markers (100bp); -ve: negative control; +ve: positive control

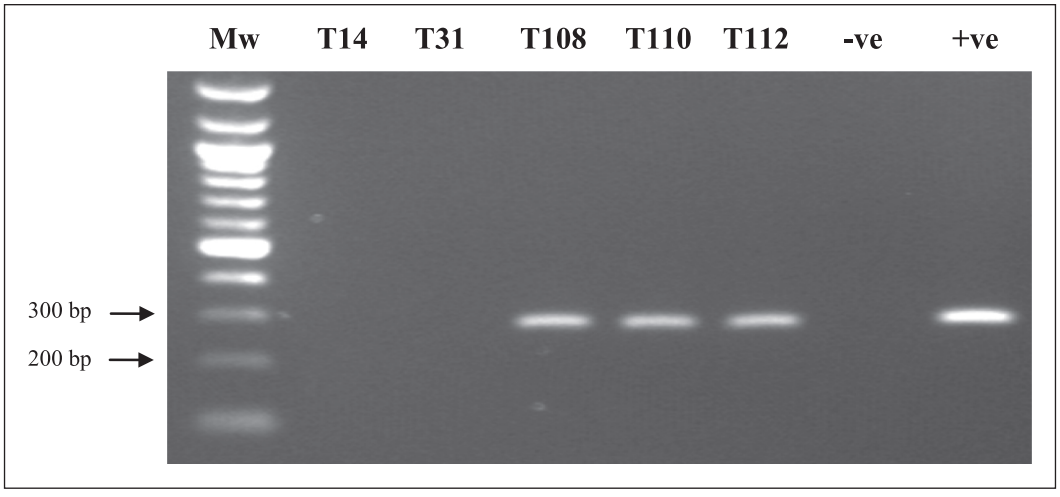

Figure 2. Detection of pathogenic leptospires on Terengganu isolates by PCR assay using G1/ G2 primer
Mw: Molecular markers (100bp); -ve: negative control; +ve: positive control

DISCUSSION

This study indicated that pathogenic leptospires were present in the Malaysian environment. From the study, Kelantan had higher detection of leptospires perhaps due to high incidence of rainfall in the area. The Northeast Monsoon usually carries heavy rainfall, particularly to the east coast states of Peninsular Malaysia. The monsoon often starts from November to March. The association of leptospirosis and rainfall during monsoon also had been reported in India (Kuriakose *et al.*, 1997). The number of leptospirosis cases in 1994 was high especially in June, July and August which Southwest monsoon usually occurred. About 557 cases have been reported during that year compared to only 21 cases in 1987 to 1988. Therefore, heavy rainfall could facilitate the spread of the organism and contaminate the camp environment.

There is now evidence that leptospires are found at both camps. Appropriate conditions such as pH of water, temperature, characteristics of the water or soil and availability of wild animals which act as reservoirs will sustain the leptospires in the environment. Based on a study in Thailand, the organism can be found in the rice fields of Nakhornratchasrima Province in Thailand when certain conditions were accessible such as stagnant water, depth ranging from 5 to 10 cm, median pH is 7.6 and median temperature is 34.5°C (Tangkanakul, 2000). Other study done by Trueba *et al*. (2004) suggested that viscosity and salt concentration also play an important role for leptospiral survival in harsh condition. They postulated that viscous solution favor a matrix that keeps cells together and facilitates translational motility for this organism.

Present study on the isolation of *Leptospira* through bacterial culture showed that the isolation percentage is quite low. The bacteriological prevalence of leptospirosis is subjected to the sensitivity of the culture technique used. Besides that, the degree of success in isolating leptospires is affected by a few factors such as the number of inoculation made, the type of medium used, and the duration for which the cultures are kept. Many strains of pathogenic leptospires from water and soil could be obtained by upgrading the previously established method of leptospires isolation. On the other hand, bacteriological culture is a insensitive method for diagnosis purposes (Safiulah *et al.*, 2009). This is possibly due to slow growing leptospires, thus culture does not contribute to rapid diagnosis in early phase of disease and by the time culture is detected as positive the patient had developed IgG antibodies against leptospirosis.

Comparison between 8-Azaguanine test and PCR will give presumptive classification regarding the isolates. Three groups can be made from both test result. For the first group, the result of 8-Azaguanine test was negative while PCR gave positive. Thus, the isolate can be confirmed as pathogenic leptospires. The isolate is considered as saprophytic and fall into the second group when the PCR gave negative result and positive result for 8-Azaguanine. However, when PCR and 8-Azaguanine gave positive result, it goes into third group which showed doubtful result. Doubtful result had occurred possibly due to coexistence of pathogenic and saprophytic leptospires in the natural water (Murgia *et al.*, 1997). They stated that false negative result would be easily misinterpreted as saprophytic leptospires which are able to grow faster and have similar morphology with pathogenic ones. The proportions of 3 groups were as followed: first group gave 20%, the third group showed 80% and none for second groups.

The findings of this study showed that detection of leptospires using molecular techniques were more dependable compared to conventional method. It can be seen from this present result that the percentage of pathogenic leptospires was higher with the use of PCR assay than by the use of 8-Azaguanine inhibition test. Perolat *et al.* (1994) reported that with the result that they had, it emphasized the practical value of PCR-based techniques in

classifying *Leptospira* isolates at species level and also intraspecific population structures studies. They added that old methods for identification of *Leptospira* such as growth response to 8-azaguanine, lipase activity and ability to grow at incubation temperature of 13, 30 and 37ºC were not reliable for species identification. Other researchers urge that better and sensitive methods such as PCR need to be established to eliminate false positive results (Kuriakose *et al.*, 2008).

Based on MAT serovar screening against 15 leptospiral isolates, it gave a low titre to *L. interrogans* serovars *hebdomadis* (1:100) for two Terengganu isolates and none for Kelantan isolates. Therefore, other set of reference hyperimmune sera are needed to cover other serovars (Vijayachari *et al.*, 2007).

In conclusion, further tests are needed to identify the other leptospiral isolates. Presence of pathogenic leptospires for prolonged period of time in the environment could cause leptospirosis outbreak. Therefore, precaution should be taken especially by camp management to ensure the camp and the environments are safe for the trainees.

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REFERENCES

Alexander, A.D., Evans, L.B., Baker, M.F., Baker, H.J., Ellison, D. & Marriapan, M. (1975). Pathogenic leptospires isolated from Malaysian surface waters. *Applied and Environmental Microbiology* **29**(1): 30–33.

Bahaman, A.R. & Ibrahim, A.L. (1988). A review of leptospirosis in Malaysia. *Veterinary Research Communications* **12**(2): 179–189.

Bahaman, A.R., Ibrahim, A.L. & Adam, H. (1987). Serological prevalence of leptospiral infection in domestic animals in West Malaysia. *Epidemiology and Infection* **99**(2): 379–392.

Cole, J.R., Sulzer, C.R. & Pursell, A.R. (1973). Improved microtechnique for the leptospiral microscopic agglutination test. *Applied and Environmental Microbiology* **25**(6): 976–980.

Diesch, S.L., Mcculloch, W.F., Braun, J.L. & Crawford, R.P. (1969). Environmental studies on the survival of leptospires in a farm creek following a human leptospirosis outbreak in Iowa. *Journal of Wildlife Diseases* **5**(3): 166–173.

Faine, S., Adler, B., Bolin, C. & Perolat, P. (1999). *Leptospira and leptospirosis* (2nd Edition ed.) CRC Press Boca Ratón, Florida.

Gordon, L.M. (1977). *Leptospira interrogans* serotype *hardjo* outbreak in a Victorian dairy herd and associated infection in man. *Australian Veterinary Journal* **53**(5): 227–229.

Gravekamp, C., Van de Kemp, H., Franzen, M., Carrington, D., Schoone, G.J., Van Eys, G., Everard, C.O.R., Hartskeerl, R.A. & Terpstra, W.J. (1993). Detection of seven species of pathogenic leptospires by PCR using two sets of primers. *Microbiology* **139**(8): 1691.

Issazadeh, K., Amirmozaffari, N., Mehrabian, S. & Oryan, H. (2009). Assessment of distribution *Leptospira spp*. in surface waters of Guilan province. *World Journal of Zoology* **4**(2): 79–84.

Johnson, R.C. & Harris, V.G. (1967). Differentiation of pathogenic and saprophytic leptospires I. growth at low temperatures. *Journal of Bacteriology* **94**(1): 27–31.

Johnson, R.C. & Rogers, P. (1964). Differentiation of pathogenic and saprophytic leptospires with 8-azaguanine. *Journal of Bacteriology* **88**(6): 1618–1623.

Khairani-Bejo, S. (2001). Epidemiology of *Leptospira interrogans* serovars *hardjo* infection in cattle. PhD Thesis. Universiti Putra Malaysia. p: 36–83.

Kuriakose, M., Eapen, C.K. & Paul, R. (1997). Leptospirosis in Kolenchery, Kerala, India: Epidemiology, prevalent local serogroups and servoars and a new serovar. *European Journal of Epidemiology* **13**(6): 691–697.

Kuriakose, M., Paul, R., Joseph, M.R., Sugathan, S. & Sudha, T.N. (2008). Leptospirosis in a midland rural area of Kerala state. *The Indian Journal of Medical Research* **128**(3): 307.

Luchini, D., Meacci, F., Oggioni, M., Morabito, G., D'Amato, V., Gabbrielli, M. & Pozzi, G. (2008). Molecular detection of *Leptospira interrogans* in human tissues and environmental samples in a lethal case of leptospirosis. *International Journal Legal Medicine* **122**(3): 229–233.

Murgia, R., Riquelme, N., Baranton, G. & Cinco, M. (1997). Oligonucleotides specific for pathogenic and saprophytic *Leptospira* occurring in water. *FEMS Microbiology Letters* **148**(1): 27–34.

Perolat, P., Merien, F., Ellis, W.A. & Baraton, G. (1994). Characterization of *Leptospira* isolates from Serovar Hardjo by Ribotyping, Arbitrarily Primed PCR, and Mapped Restriction Site Polymorphisms. *Journal of Clinical Microbiology* **32**(8): 1949–1957.

Safiulah, S.A., Saleh, A.A. & Munwar, S. (2009). Laboratory methods for diagnosing leptospirosis: A review. *Bangladesh Journal of Medical Microbiology* **3**(1): 39.

Tangkanakul, W. (2000). Risk factors associated with leptospirosis in northeastern Thailand, 1998. *The American Journal of Tropical Medicine and Hygiene* **63**(3): 204–208.

Trueba, G., Zapata, S., Madrid, K., Cullen, P. & Haake, D. (2004). Cell Aggregation: A mechanism of pathogenic *Leptospira* to survive in fresh water. *International Microbiology* **7:** 35–40.

Vijayachari, P., Sharma, S. & Natarajaseenivasan, K. (2007). Serological Characterization of Leptospires. *WHO Leptospirosis Laboratory Manual* 46–51.

Wilson, R. & Fujioka, R. (1995). Development of a method to selectively isolate pathogenic leptospira from environmental samples. *Water Science and Technology* **31**(5): 275–282.