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LEPTOSPIRAL INFECTION IN STRAY DOGS IN MALAYSIA

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ABSTRACT

Leptospirosis is a zoonotic disease of worldwide importance. Besides rodents, dogs have also been implicated as a source of infection to humans. Samples of urine and blood from 142 stray dogs were examined for leptospirosis by serological tests and bacterial cultures. Of these, 83% (118/142) and 33% (47/142) of the serum samples were positive by ELISA-IgM/IgG and the microscopic agglutination test (MAT) respectively. No leptospiral isolates were obtained on culture. *Leptospira interrogans* serovar *pomona* was found to be the most predominant serovar in the dogs with an infection rate of 68% at MAT titres of ≥ 100. Urine and serum samples were randomly selected for detection and identification of leptospires by the polymerase chain reaction (PCR) and low-stringency PCR assay respectively. All seropositive samples selected were positive by PCR assay. One urine sample showed the presence of leptospires based on PCR assay.

Keywords: Leptospirosis, serological prevalence, dogs, PCR

INTRODUCTION

Leptospirosis, a worldwide zoonotic disease, affects domestic animals, wildlife and humans. This disease, of public health importance, has recently been recognised as a re-emerging disease particularly in tropical countries. Transmission of infection is often via indirect contact with water, moist soil or food that had been contaminated with urine of infected animals (Bahaman and Ibrahim, 1987; Bovet *et al.*, 1999; Prescott *et al.*, 2002). Natural reservoirs of infection are rodents and domestic animals including cattle, pigs and dogs (Bahaman and Ibrahim, 1988; Prescott *et al.*, 2002). Bahaman and Ibrahim (1988) excluded stray dogs in their study. As a result, the information on leptospiral infection in Malaysian dogs is still lacking. Dogs can be an important source of infection to humans because of their close association with people and their unsanitary habits. The aim of this study is to determine the prevalence of leptospiral infection in stray dogs and the appropriate assay to identify the infection. The findings would provide information on the role of stray dogs as a carrier of leptospires and the risk that they may pose to the human population at large.

MATERIALS AND METHODS

Test animals and samples

One hundred and forty-two stray dogs were randomly selected. Two to three milliliters of blood sample were collected from the cephalic vein of each dog. A few drops of whole blood were immediately cultured in Johnson-Seiter (JS) semi-solid medium containing 200 µg/ml of 5-fluorouracil. The rest of the blood was allowed to clot and the serum obtained by centrifugation at 3,500 rpm for 10 minutes and stored at –20 °C until required for serological tests and/or PCR.

A 10 ml volume of urine sample was collected from each dog and a few drops of undiluted urine were cultured for leptospires. A portion of the urine sample was diluted (two serial 10-fold dilutions) with the liquid medium. Again, one or two drops of each dilution were transferred into each of four tubes containing 3 ml of JS semi-solid medium with 200 µg/ml or 400 µg/ml of 5-fluorouracil. The rest of the urine sample from each dog was transferred into a 15 ml centrifuge tube containing 2.5 ml of 100 mM EDTA, pH 8.0 and 0.5% formaldehyde. The EDTA-formaldehyde urine samples were transferred to the laboratory within two hours. All samples were centrifuged at 800 rpm for 10 minutes to eliminate large particles such as bladder cells and urinary crystals or cylinders. The supernatant was transferred into a clean 15 ml centrifuge tube and centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was removed, leaving behind 100 µl of fluid. The pellet was suspended in 1 ml of 1 mM EDTA, pH 8.0. The suspension was stored at –20°C until use for DNA isolation.

The blood and urine sample cultures were observed weekly for 3 months for presence of leptospires.

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Microscopic agglutination test (MAT)

All serum samples were examined for leptospiral antibodies by the MAT as described by Cole *et al.* (1973). Serial two-fold dilutions of serum were prepared in microtiter plates. An equal volume of diluted serum and a well grown suspension of leptospires with density of 1-2 x 10^8 leptospires/ml was mixed and incubated at 30°C for 2 hours. At the end of incubation a drop from each well was examined for agglutination under darkfield microscopy.

Enzyme Linked Immunosorbent Assay (ELISA)

An enzyme-linked immunosorbent assay (ELISA) as described by Terpstra *et al.* (1985) was used as a parallel test for screening leptospiral immunoglobulin (Ig) M and IgG antibodies. Sera detected by ELISA were recorded as positive if their OD readings were two times higher than the OD readings of the negative control sera. The serum samples however were considered to be positive if their IgM titres were $\geq$160 in spite of a failure in IgG antibody detection.

DNA isolation

Urine and serum samples were randomly selected and detected for leptospiral infection by PCR. A 300 - 500 µl volume of serum or 1 ml of urine sample was centrifuged at 13,000 rpm for 20 minutes at 4°C. The pellets were washed twice with 500 µl of sterile distilled water and centrifuged again at 13,000 rpm for 10 minutes. The pellets were then suspended in 50 µl of sterile and filtered TE buffer. DNA was released by heating the sample at 95°C in vigorously boiling water for 10 minutes. The sample was immediately quenched on ice and stored at –20°C prior to PCR. For pure leptospires, 1 ml of *L. interrogans* serovar culture, with a concentration of 10^7-10^8 leptospires/ml, was centrifuged at 10,000 rpm for 20 minutes at 4°C. The pellets were washed a few times in sterile PBS, pH 7.2 by centrifugation. The pellets were then suspended in 100 µl of TE buffer and heated to release DNA contents as described above.

Polymerase chain reaction assay (PCR)

Amplification of DNA was performed in a total volume of 50 µl. The reaction mixture consisted of 5 µl of 10X PCR buffer, 1.5 µl of 50 mM $MgCl_2$ (final concentration of 1.5 mM), 1 µl of 10 mM dNTP mix (dATP, dTTP, dGTP, dCTP) at a concentration of 200 mM each of the four deoxynucleotide triphosphates. A pair of primers G1 (5'-CTG AAT CGC TGT ATA AAA GT -3') and G2 (5'- GGA AAA CAA ATG GTC GGA AG -3') was used at a final concentration of 20 pmoles. This pair of primers was proven to be able to amplify DNA by PCR from all pathogenic leptospires species, except *L. kirschneri* (Gravekamp *et al.*, 1993).

A volume of 2 – 5 µl of isolated DNA from pure leptospires or 10 ml of urine and serum samples were added as templates, and sterile distilled water was then included to a final volume of 50 µl. For negative controls, sterile distilled water was added instead of the template material. DNA amplification reactions were performed in a Thermal Cycler (Cyclogene, UK).

The specific amplification cycle consisted of an initial denaturation step at 95°C for 3 minutes, followed by 30 cycles of amplification at 94°C for 45 seconds, 50°C for 2 minutes and 72°C for 2 minutes. An additional 10 minutes at 72°C was included in the final cycle. A 10 µl of the amplified PCR products was analysed by electrophoresis in 2% (w/v) agarose gel. The gel was then stained with 0.5 mg/ml of ethidium bromide for 30 minutes and then washed in distilled water for 10 – 15 minutes. The gel was finally visualised and photographed under UV light.

Low-stringency PCR

The PCR-positive samples were then examined for serovars involved in the infection by low-stringency PCR. The same pair of primers (G1-G2) was used for amplification under low-stringency conditions. As a result, a set of low-stringency products and the specific DNA sequence defined by the primers were produced. The first cycle of amplification consisted of denaturation at 95°C for 5 minutes, annealing at 30°C for 2 minutes and extension at 72°C for 3 minutes. The second cycle was identical to the first cycle, except that the denaturation step was at 94°C for 45 seconds. The subsequent 33 cycles consisted of denaturation at 94°C for 45 seconds, primer annealing at 40°C for 1 minute and extension at 72°C for 2 minutes. An extended incubation at 72°C for 5 minutes was included at the end of the programme to ensure complete extension. The amplified products were subjected to electrophoresis on a 10% polyacrylamide gel and finally stained with silver.

RESULTS

Prevalence of leptospiral infection

Table 1 shows the prevalence of leptospiral infection in the stray dogs examined by MAT, ELISA and bacterial cultures. It can be seen that 83% of the dogs had leptospiral infection as determined by the ELISA while 33% of the dogs were positive by MAT, with MAT titres of $\geq$ 100. *Leptospira interrogans* serovar *pomona* was found to be the most predominant serovar at 68% (97/142) in the dog examined. No leptospiral isolate was obtained from the 142 specimens by bacterial cultures. It was also seen that 94% (16/17) of the PCR-positive samples had IgM antibodies with titres of $\geq$160. These

Table 1: Bacteriological and serological prevalence of leptospiral infection in the stray dogs

Tests	No. of samples tested	No. (%) of positives
MAT	142	47 (33%)
ELISA (IgM/IgG)	142	118 (83%)
Culture	284	0

Table 2: Detection for leptospiral DNA in selected seropositive samples by PCR

Dog#	Sex	MAT		ELISA		PCR	
		Titre	Serovar	IgM Titre	IgG	Blood	Urine
S1	male	50	*pomona*	80	-	+	+
S2	male	< 50	*pomona*	320	-	+	-
S3	male	-	-	640	-	+	-
S4	male	-	-	320	-	+	-
S5	male	< 50	*pomona*	320	-	+	-
S6	male	-	-	640	-	+	-
S7	male	50	*pomona*	320	-	+	-
S8	male	50	*pomona*	320	-	+	-
S9	male	400	*australis*	160	+	+	-
S10	female	-	-	1280	-	+	-
S11	female	-	-	640	-	+	-
S12	female	50	*pomona*	640	-	+	-
S13	female	100	*pomona*	640	-	+	-
S14	male	-	-	320	-	+	-
S15	male	100	*pomona*	320	-	+	-
S16	male	50	*pomona*	320	-	+	-
S17	female	-	-	320	-	+	-

findings showed that PCR and ELISA IgM were able to detect the early phase of infection where leptospiraemia was still prevalent (Table 2).

Identification of leptospiral serovars

Specific amplification of leptospiral serovars: *icterohaemorrhagiae*, *pomona*, *canicola*, *australis*, *hardjo*, *copenhageni* and *portlandvere* with primers G1-G2 showed major DNA products of approximately 290 bp (Figure 1). These findings were similar to the results reported by Gravekamp *et al.* (1993), Caballero *et al.* (1994) and Brown *et al.* (1995).

The PCR-positive samples were amplified using the same pair of primers G1-G2 under low-stringency conditions. The results are shown in Figure2. A complex set of low-stringency products and the specific amplification products were generated and conserved in all cases. The results helped to verify that the DNA amplified is indeed derived from pathogenic leptospiral species. The specific amplicon of approximately 290 bp was seen to be the most intense component of the low-stringency PCR profiles for serovars: *icterohaemorrhagiae*, *canicola* and *australis*. Serovar *pomona* had the most intense band of 290 bp. Other serovars had differed slightly from each other. These observations were consistent and reproducible following repeated tests. The unknown serovars detected in the dog specimens showed DNA profiles closely related to serovar *pomona*. However, they also shared a few more bands (240 bp and 450 bp) as seen in DNA profiles of serovars *canicola* and *icterohaemorrhagiae*.

DISCUSSION

Previous studies on canine leptospirosis revealed that *serovars Leptospira interrogans, icterohaemorrhagiae* and *canicola* were predominant among the dog populations in Malaysia (Bahaman and Ibrahim, 1988). In this present study, it was shown that *L. pomona* was the most prevalent serovar among the stray dogs examined. This finding was consistent with other recent reports that serovar *pomona* is the important cause of canine leptospirosis (Birnbaum *et al.*, 1998; Prescott *et*

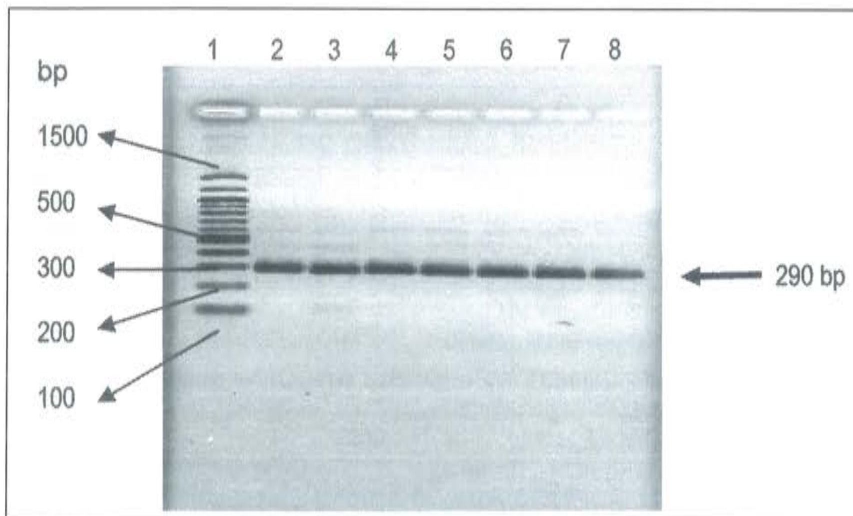

Figure 1: Ethidium bromide stained 2% agarose gel showing the specific PCR products amplified with primers G1-G2. 100 bp DNA ladder (lane 1) and the following *L. interrogans* serovars: *icterohaemorrhagiae* (lane 2); *pomona* (lane 3); *canicola* (lane 4); *australis* (lane 5); *hardjo* (lane 6); *copenhageni* (lane 7); and *portlandvere* (lane 8).

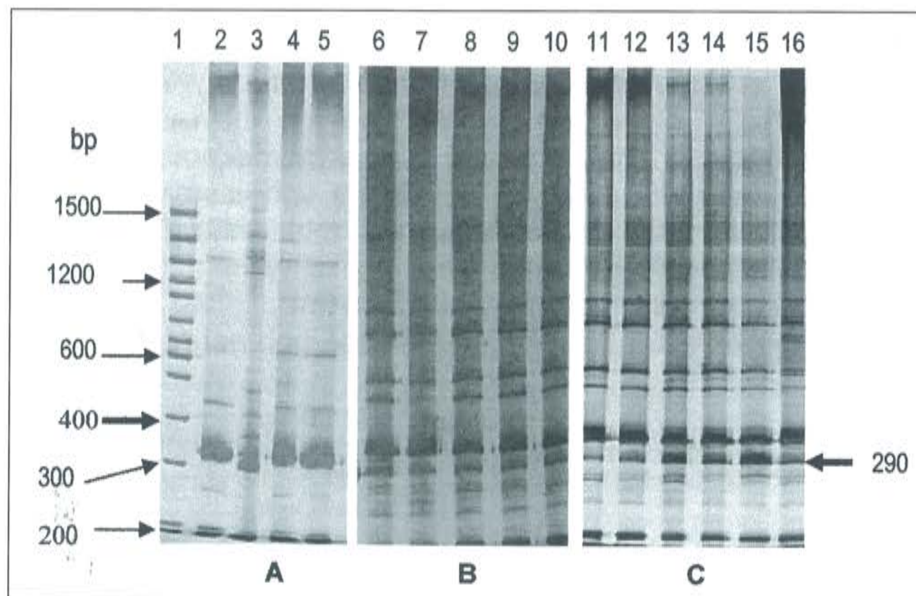

Figure 2: Silver-stained 10% polyacrylamide gels showing the LS-PCR products derived using primers G1-G2. (A) four reference *L. interrogans* serovars: *icterohaemorrhagiae* (lane 2), *pomona* (lane 3), *canicola* (lane 4), *australis* (lane 5). (B,C) unknown serovars detected in serum samples of the stray dogs (lanes 6 – 16). 100 bp DNA ladder (lane 1).

al., 2002). Infection due to serovar *hardjo* was not evident in this survey while infection due to serovar *australis* was low. The emergence of *L. pomona* infection in the stray dogs could be due to exposure to pigs, the main reservoir for serovar *pomona,* or indirectly with contaminated water or soil with urine of infected animals.

The polymerase chain reaction has shown its practical value for early diagnosis of leptospirosis, especially before IgG antibodies are detectable. This is very important in the treatment of infection and critical in limiting the spread of the disease. It was also shown to be specific for leptospires, sensitive and rapid compared to bacterial culture and serological examination. Preparation of the sample, performance of the assay and the PCR results could be obtained within one working day. Failure of leptospires to grow in cultures was observed in this study. This might be due to the fastidious nature of the organisms, the phase of infection, and pH

of urine during sample collections. High antibody titres in serum samples could also greatly reduce the possibility of isolating leptospires in cultures.

The PCR-positive samples were examined by low-stringency PCR with specific primers for identification of leptospiral serovars. All of the unknown serovars from the PCR-positive samples produced no significant difference in low-stringency PCR profiles, but they showed good agreement with *L. pomona* in this study. It can be seen that the low-stringency PCR profiles of these unknown serovars shared bands generated by *L. pomona* and *L. canicola* or *L. icterohaemorrhagiae*. These results demonstrate that the Leptospira bivalent bacterins containing serovars *icterohaemorrhagiae* and *canicola* does not protect dogs from infection by other serovars due to the specificity of the immunity. It could be concluded that our local unknown serovars might be genetic intermediates between serovar *pomona* and the prevalent serovars in the dog population such as *L. icterohaemorrhagiae and canicola.*

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