A RAPID METHOD FOR THE DETECTION OF LEPTOSPIRES IN CLINICAL SPECIMENS FROM DOGS

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SUMMARY

The microscopic agglutination test (MAT), IgM and IgG enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) assay and bacterial isolation were evaluated to detect leptospiral infection in 29 stray dogs. Results obtained showed that the PCR assay revealed 72% (21/29) of the dogs had evidence of leptospiral infection whilst ELISA and MAT showed 59% (17/29) and 10% (3/29), respectively. Leptospires were not seen in any of the bacterial cultures. It was observed that almost all of the PCR-positive dogs had IgM antibodies with titres of ≥ 160. One dog had a titre of 80 whilst three other dogs had no detectable antibodies. It was also shown that the PCR assay was more sensitive, specific and rapid method compared to the other three methods.

Keywords: leptospiral infection, PCR, diagnosis, dogs

INTRODUCTION

Leptospirosis occurs in both wild and domestic animals which, directly or indirectly, can be a source of infection to humans. Accurate and rapid determination of the source of infection is essential in limiting the spread of the disease. In addition, early diagnosis is particularly important for treatment which can then be instituted at the early stage of infection. Since leptospirosis is a common cause of acute febrile disease in tropical countries, its clinical symptoms are often confused and it may be misdiagnosed with many common febrile diseases such as dengue fever, malaria, typhoid and viral hepatitis (Levett and Whittington, 1998). The polymerase chain reaction (PCR) assay has become an ideal diagnostic test due to its sensitivity, specificity and rapid detection of small number of leptospires in clinical samples. In addition, leptospiral DNA can be detected at the initial stage of infection and even months later. Detection of leptospires in serum, urine, cerebrospinal fluid (CSF) and plasma of patients with leptospirosis by PCR assay has been reported (Merien *et al.*, 1992; Bal *et. Al.*, 1994; Brown *et al.*, 1995). Detection of leptospires in urine by PCR assay has also been successfully applied in cattle (Gerritsen *et al.*, 1991). In this study, the PCR assay was evaluated in the detection of leptospires from dog specimens (serum, plasma, and urine) and compared the results with those of the ELISA, MAT and bacterial isolation.

MATERIALS AND METHODS

Test animals

Blood and urine samples were obtained from 29 stray dogs that were kept in a non-governmental organization.

Urine samples

Approximately 11 – 12 ml of urine samples were collected from each dog. One to two drops of fresh undiluted and diluted (two serial 10-fold dilutions) urine samples were cultured in 3 ml of Johnson and Seiter (JS) semi-solid medium containing 200 or 400 mg/ml of 5-fluorouracil (5FU) for leptospiral isolation. The rest of the urine sample (10 ml) was poured into a 15 ml centrifuge tube containing 2.5 ml of 100 mM EDTA, pH 8.0 and 0.5% of formaldehyde (UNIVAR). The urine samples with EDTA-formaldehyde were then transported to the laboratory within 2 hours. All samples were centrifuged at 800 rpm for 10 minutes to eliminate large particles such as urinary bladder cells, crystals or cylinders (Merien *et al.*, 1992). 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 then removed and discarded. The pellet was suspended in 1 ml of 1 mM EDTA, pH 8.0 and transferred into a 1.5 ml microcentrifuge tube. The suspension was then centrifuged at 13,000 rpm for 10 minutes at room temperature. The supernatant was removed and discarded, leaving behind 100 ml of fluid. The pellet was washed once with 1 ml of sterile distilled water by centrifugation. The pellet was resuspended in 100 ml of the remaining fluid. The suspension was then heated at 95°C in boiling water for 10 minutes to release the DNA. As a first denaturing step, the sample was quenched on ice to destroy any residual enzymatic activity (Van Eys *et al.*, 1989). The sample was stored at –20°C until further use. In addition, some urine samples were processed for DNA isolation by using Wizard" Genomic DNA Purification Kit (Promega, USA).

Blood samples

Two to three millilitres of blood sample were collected from the cephalic vein of each dog. A few drops of whole

blood were immediately cultured in JS semi-solid medium, containing 200 mg/ml of 5-fluorouracil. Approximately, 2 ml of blood was allowed to clot in a clean tube at room temperature. The serum was obtained by centrifugation at 3,500 rpm for 10 minutes. 500 ml of serum was stored at –20°C for serological tests. The microscopic agglutination test (MAT) was performed as described by Cole *et al.* (1973) whilst the ELISA was as outlined by Terptra *et al.* (1985). The rest of the serum was then centrifuged at 13,000 rpm for 20 minutes at 4°C. The pellet was washed twice with 500 ml of sterile distilled water and centrifuged again at 13,000 rpm for 10 minutes. The pellet was then suspended in 50 ml of sterile and filtered TE buffer. The DNA was released by heating the sample at 95°C in vigorously boiling water for 10 minutes. The sample was immediately cooled on ice, and stored at –20°C prior to PCR assay. For detection of leptospires in plasma by PCR assay, another 1 ml of blood was transferred into an EDTA tube to prevent clotting. The non-coagulated blood sample was then processed for DNA using Wizard" Genomic DNA Purification Kit (Promega, USA).

During the exponential growth phase which has an approximate concentration of $10^7 - 10^8$ leptospires/ml, a 10 ml volume of each leptospiral serovar culture was transferred into a clean 15 ml centrifuge tube and centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was discarded. The pellet was suspended and washed a few times in sterile PBS, pH 7.2 by centrifugation. The cells were resuspended in the remaining fluid. The DNA was isolated by boiling as described earlier or using Wizard" Genomic DNA purification Kit (Promega, USA).

Polymerase Chain Reaction Assay

Amplification of the DNA was performed in a 50 ml reaction mixture consisting of a 5 ml of 10 X PCR buffer (Biotools, Spain), 1.5 ml of 50 mM $MgCl_2$ (final concentration of 1.5 mM), 1 ml of Taq DNA Polymerase (1 unit/ml) (Biotools, Spain), and 1 ml of 10 mM dNTP mix (dATP, dTTP, dGTP, dCTP) (Biotools, Spain) at a concentration of 200 mM for each of the four deoxynucleotide triphosphates. A pair of primers: G1 (5^1 - CTG AAT CGC TGT ATA AAA GT - 3^1) and G2 (5^1 - GGA AAA CAA ATG GTC GGA AG - 3^1) (GIBCO BRL, USA) was used at a final concentration of 20 pmoles. This pair of primers have been proven to amplify DNA from all pathogenic leptospiral serovars, except L. kirschneri (Gravekamp et al., 1993; Caballero et al., 1994; Brown et al., 1995). A volume of 2 – 5 ml of DNA extract from reference leptospiral serovars were taken as positive controls and 10 ml of DNA from urine, blood, or serum samples were added as template and sterile distilled water was added to give a final volume of 50 ml. 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 is made up 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 ml of the amplified PCR products was analysed by electrophoresis in a 2% (w/v) agarose gel in 1X TBE buffer. The gel was then stained with 0.5 mg/ml of ethidium bromide for 30 minutes and washed in distilled water for another 10–15 minutes. The gel was finally visualised and photographed under UV light.

Table 1. Incidence of leptospiral infection in 29 stray dogs based on four diagnostic methods

Methods	No. of positives	% of positives
Culture	0	0%
MAT	3	10%
ELISA	17	59%
PCR	21	72%

RESULTS

Out of the 29 stray dogs, 21 dogs were positive by the PCR assay. Amongst the PCR positive dogs, 17 had IgM antibodies with titres of ≥ 160 as indicated by ELISA. Three of the dogs were also positive on MAT with titres of ≥ 100. No isolate was obtained on cultures (Table 1).

Specific Polymerase Chain Reaction Assay

Specific amplification of leptospiral serovars: *icterohaemorrhagiae, pomona, canicola, australis, hardjo, copenhageni* and *portlandvere* with primers G1-G2 showed a major DNA product of approximately 290 bp. These findings have been similarly reported by Gravekamp *et al.* (1993), Caballero *et al.* (1994), and Brown *et al.* (1995). The DNA extracted from blood samples by Wizard" Genomic DNA Purification Kit (Promega) were positive as indicated by the DNA product of 290 bp. (Fig. 1). Similarly, the DNA extracted directly from the same blood samples by boiling were also positive.

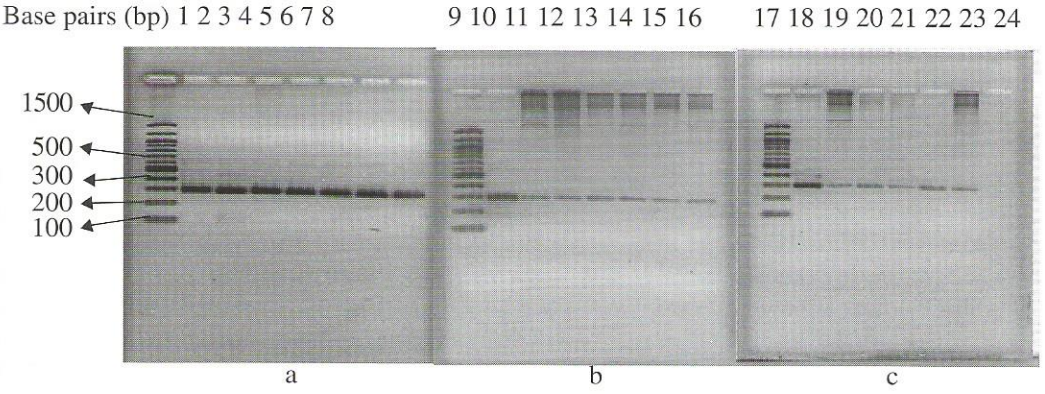

Fig. 1. Ethidium bromide stained 2% agarose gel showing the specific PCR products amplified with primers G1-G2: 100 bp DNA ladder (BIO-RAD) (lanes 1, 9 and 17) and the following (a) ***L. interrogans*** **serovars: *icterohaemorrhagiae* (lane 2); *pomona* (lane 3); *canicola* (lane 4); *australis* (lane 5); *hardjo* (lane 6); *copenhageni* (lane 7); and *portlandvere* (lane 8).** (b) **serovar *icterohaemorrhagiae* (lane 10); unknown serovars detected in whole blood samples from dog Nos. 13, 14, 15, 18, 20 and 21 (lanes 11 – 16, respectively).** (c) **serovar *icterohaemorrhagiae* (lane 18); unknown serovars detected in whole blood samples from dog Nos. 23, 16, 17, 19 and 22 (lanes 19 - 23, respectively); and negative control (lane 24).**

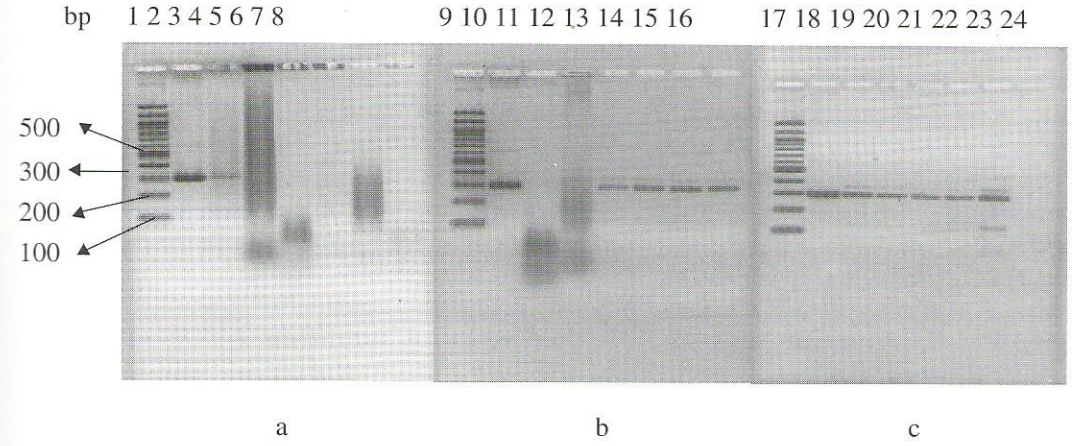

Fig. 2. Ethidium bromide stained 2% agarose gels showing the bands of the expected size by using primers G1-G2: 100 bp DNA ladder (lanes 1, 9 and 17). (a) **serovar *canicola* (lane 2); unknown serovar detected in urine of the dog No.2 (lane 3); The rest of urine specimens (lanes 4 – 8) from dog Nos. 3, 5, 6, 7 and 8 were negative for leptospires.** (b) **serovar *canicola* (lane 10); urine samples from dog Nos. 9 and 10 (lanes 11 and 12, respectively); unknown serovars detected in sera samples from dog Nos. 2, 3, 5 and 6 (lanes 13 – 16, respectively).** (c) **serovar *australis* (lane 18); unknown serovars detected in sera samples from dog Nos. 7, 8, 9, 10 and 11 (lanes 19 – 23, respectively); negative control (lane 24).**

DISCUSSION

Availability of a sensitive, specific and rapid method for the diagnosis of leptospirosis during the early stage of infection is very important in the treatment of the disease in animals and humans. In dogs, early detection also provides an opportunity to reduce or prevent the spread of infection to humans. The PCR assay has proven to be valuable in the direct detection of leptospires in clinical materials (Gerritsen *et al.*, 1991; Merien *et al.*, 1992; Brown *et al.*, 1995). Out of the twenty-nine specimens obtained from the dogs surveyed, twenty blood and one urine samples were PCR positive. The pair of primers, G1-G2, was able to detect and amplify the selected pathogenic leptospiral DNA except *L. kirschneri* as well as from the clinical samples. All twenty-one PCR-positive samples contained amplified leptospiral DNA fragments of approximately 290 bp.

The detection of leptospires by PCR assay at an early stage of infection is valuable in the diagnosis of leptospirosis. Other methods often failed or were proven to be unreliable. It is seen in this study that the PCR results were closely correlated with the IgM antibodies being detected by ELISA. On the other hand, the MAT was specific but it was not useful during the early stage of the disease when antibody to leptospires has not developed or

was present at a low level in the serum. In addition, MAT requires the use of a large number of live serovars as antigens. It was seen that PCR was more sensitive than either MAT or cultures for the detection of leptospirosis.

The PCR showed positive results in some sero-negative samples. This showed that the assay was able to detect leptospires in blood samples even before the development of antibodies. This indicated the high sensitivity of PCR. Positive PCR results in culture-negative samples suggest that PCR is also sensitive enough to detect and amplify DNA from dead and/or lysed leptospires. The fastidious nature of the organisms, the phase of infection and pH of urine during sample collection often contribute to failure in culturing leptospires.

It was shown from this study that the PCR assay was more reactive to DNA from sera and plasma (whole blood) than urine samples. The urine samples were negative which may be due to a number of reasons. It could be due to the number of leptospires present in the urine samples was below detectable level or it could be due to the presence of inhibitory factors in the urine samples or perhaps, the animals were not shedding leptospires anymore.

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RINGKASAN

CARA CEPAT BAGI MENYIASAT LEPTOSPIRA DALAM SPESIMEN KLINIK DARIPADA ANJING

Ujian penggumpalan mikroskopi (MAT), asai enzim-berangkai penjerap-imun (ELISA) IgM dan IgG, asai gerakbalas rantaian polymerase (PCR) dan pengasingan bakteria di nilai bagi penyiasatan jangkitan leptospira pada 29 ekor anjing liar. Keputusan yang di perolehi menunjukkan asai PCR mendedahkan 72% (21/29) anjing mendapat jangkitan leptospira sementara ELISA dan MAT masing-masing menunjukkan 59% (17/29) dan 10% (3/29) anjing terjangkit. Leptospira tidak kelihatan pada sebarang kultura bakteria. Hampir keseluruhan anjing yang positif bagi ujian PCR mempunyai antibodi IgM dengan titer $\geq$ 160. Satu ekor anjing mempunyai titer 80 sementara 3 ekor anjing lagi di kesan tidak mempunyai antibodi. Jelaslah, asai PCR adalah lebih sensitif, khusus dan ianya merupakan cara yang cepat berbanding dengan tiga cara yang lain.