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Application of Polymerase Chain Reaction Assay in Detection of *Leptospira icterohaemorrhagiae* in Experimentally Infected Mice

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Abstract: A total of 24 mice were inoculated with 1×10^7 of *Leptospira icterohaemorrhagiae.* Four mice as negative control were not inoculated. Four inoculated mice were sacrificed at each sampling day on day 1, 3, 7, 10, 14 and 21 post inoculation. Blood and kidney samples were collected and inoculated into JS medium for isolation of *L. icterohaemorrhagiae.* Polymerase Chain Reaction (PCR) assay was performed to detect the present of the organisms in blood and kidneys. All culture samples were negative for *L. icterohaemorrhagiae*. However, one (1/24) blood sample was positive by PCR. It is apparent that detection of leptospires in blood with PCR is a promising approach for early diagnosis of leptospirosis.

Key words: Blood, kidney, PCR, *Leptospira icterohaemorrhagiae*

INTRODUCTION

The presence of Leptospira icterohaemorrhagiae infection or leptospirosis in Malaysia was originally noted by Fletcher in 1928 who demonstrated infection in man, dogs and several species of rats. Humans are infected through contact with animal reservoirs or a contaminated environment such as soil, sewage, or water. Several animal species especially rats are natural reservoirs of the disease, while humans are a dead-end host. Laboratory diagnosis of leptospirosis is predominantly achieved either by isolation of the causative organism or by serological tests. Bacterio-logical methods for the isolation of pathogenic organisms are slow, labor intensive and often unsuccessful. Serological tests have been used extensively to determine whether an animal is, or has been infected and are considered to be the most beneficial laboratory tool for diagnosis of leptospirosis. The Microscopic Agglutination Test (MAT) using live antigen is considered the international standard method for the detection of leptospiral antibodies. However, the MAT has many disadvantages, because it requires the use of several leptospiral serovars in their active growth phase. The maintenance and standardization of such live antigens is difficult, expensive and tedious and time consuming (Vanasco *et al*., 2001). Nowadays, molecular diagnostic methods are increasingly being used for clinical diagnostic in endemic areas because of their sensitivity and specificity. PCR amplification techniques should help to characterize any *Leptospira* DNA sequences present, especially in an early stage of infection (Oliveira *et al*., 2003). Thus, the objective of this project is to evaluate the PCR as a diagnostic tool for early detection of leptospiral infection.

MATERIALS AND METHODS

Experimental animals: A total of 24 mice, aged 6 week-old and weighing 200 g were inoculated with 1 mL of 1×10^7 of *L. icterohaemorrhagiae* intraperitoneally whilst 4 mice were used as negative controls and remained uninoculated. Four mice were sacrificed at day 1, 3, 7, 10, 14 and 21 post inoculations. Blood and kidney samples were collected for isolation of the organisms and PCR assay.

Bacterial isolation: Two drops of blood were inoculated directly into a JS semi solid media. Kidney was crushed with syringe into JS liquid media and mix. The mixture was allowed to stand at room temperature for 15 min. Two drops of the supernatant were then transferred into the JS semi solid media. Inoculated media were incubated at 30°C incubator for 12 weeks and checked under dark field microscope weekly starting from week 3 post inoculation to check for the presence of *L icterohaemorrhagiae*.

Polymerase Chain Reaction (PCR) assay: The DNA of *L. icterohaemorrhagiae*, blood and kidneys were extracted using Wizard® Genomic Purification Kit

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(Promega, USA). Two sets of primers for PCR, G1 and G2 were used (amplification product 285-bp). Reactions were performed in PCR buffer containing 3.0 mM $MgCl_2$, 0.2 mM dNTP, 1.25 units *Taq* polymerase, 0.68 µM primers (G1 and G2) and 5 µL DNA templates and the mixtures were placed in PCR thermal cycler. One amplification cycle consisted of denaturation for 1 min at 95°C, anneling of primers for 1 min at 55°C and elongation for 1.5 min at 72°C. Thirty eight amplification cycles were used and the last elongation step was extended to 10 min. After amplification, a 10 µL of PCR product were subjected to electrophoresis on a 1% agarose gel in 1×TBE buffer at 65 V for 1.5 h, stained with ethidium bromide (5 mg mL^{-1}) and photographed.

RESULTS AND DISCUSSION

All blood and kidneys samples were negative for *Leptospira* in isolation method. One blood sample was positive to *L. icterohaemorrhagiae* by PCR assay whilst all kidney samples were negative (Fig. 1). The PCR product was located approximately at 285bp.

Several studies have applied PCR assay for the amplification of *Leptospira* DNA by using either well-conserved primers, selected within the sequence of the *Leptospira* rRNA 16S gene or primers which are specific for one or a few serovars. The present study explored the use of PCR analysis of blood and kidneys as a diagnostic tool for leptospirosis by application of a procedure involving DNA extraction and subsequent PCR analysis of mice blood and kidney samples taken at different stages of the disease. Polymerase chain reaction assay was able to detect leptospires in blood samples which was negative by culturing method. Thus, PCR is a more sensitive and rapid alternative to culturing for direct detection of leptospires in blood. A positive PCR outcome is actually only evidence of the presence of leptospiral DNA, which may originate from both viable and dead bacterial in a sample. Leptospires do not survive long in clinical sample such as blood or urine. The better results obtained by PCR than by culturing may thus be attributed to the fact that PCR detected dead bacteria as well.

The present study showed that, leptospires were only detected in blood samples at day 3 post inoculation. This finding contrary to a previous study which managed to isolate leptospires from blood from day 2-4 post inoculations (Palaniappan *et al*., 2005). The low detection results might be due to several factors such as the inoculum used to inoculate the mice. The inoculum was from the laboratory strain and was sub cultured many times that probably, it has lost its pathogenicity Therefore, the ability of the bacteria to multiply in the

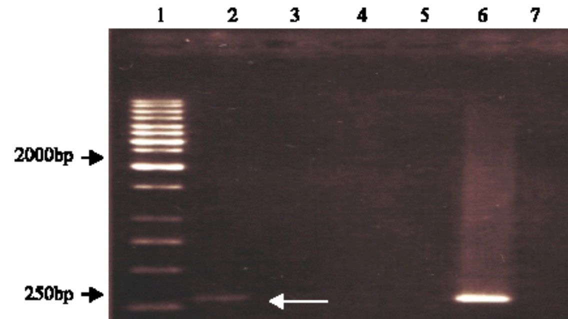

Fig. 1: Polymerase chain reaction result of blood sample at Day 14 post inoculation. From left to right: Lane 1: 1 kb marker; lane 2: Mice 1; lane 3: Mice 2; lane 4: Mice 3; lane 5: Mice 4; lane 6: Positive control; lane 7: Negative control

blood might have reduced markedly. Furthermore, the presence of heme compound in complete hemolized blood during DNA extraction could inhibit the PCR (Navarro *et al*., 2004).

CONCLUSION

In conclusion, PCR described in this present study is shown to be a promising technique for early diagnosis of leptospirosis. Polymerase chain reaction assay has proven to be more sensitive and rapid than culturing method.

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