COMPARISON OF BACTERIOLOGIC CULTURE, DARK FIELD MICROSCOPY AND PCR FOR DETECTION OF *Leptospira* IN BIOMATERIALS*

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ABSTRACT

A study was conducted in the Department of Microbiology, COVAS, Mannuthy in which suspected clinical samples and biomaterials from rodents were subjected to bacteriologic culture, Dark field microscopy and PCR for detection of leptospires. Bacteriologic culture and DFM could be performed only on freshly collected samples where as PCR could detect leptospires even in long preserved ones. DFM or culturing were found to be either unreliable or too slow to contribute to an early diagnosis compared to PCR. At the same time DFM was very useful to detect presence of leptospires in cultures and to check the purity of cultures. Though not suitable for routine use and early diagnosis the advantage of isolation was that any serovar could be detected and kept for future identification.

INTRODUCTION

The wide spectrum of clinical signs from mild to fatal forms make the diagnosis of leptospirosis difficult. The diagnostic approaches include direct microscopic demonstration of organism, isolation, serology and molecular techniques for detection of nucleic acid A study was conducted in the Department of Microbiology, COVAS, Mannuthy in which suspected clinical samples and biomaterials from rodents were subjected to bacteriologic culture, Dark field microscopy and PCR for detection of leptospires.

MATERIALS AND METHODS

Clinical samples

One hundred and seventeen including serum/plasma (92), urine (18), kidney tissues (5) and milk (samples 2) were collected from suspected cases of canine/bovine/caprine/human leptospirosis.

Bio-materials from rodent reservoirs

The kidney tissues (25) and urine (5) were collected from 21 rats and 4 bandicoots captured from the neighbouring farm premises

Dark Field Microscopy (DFM)

The urine and blood samples collected fresh from suspected cases and urine from Rodents were first subjected to DFM.

Blood

Two milliliters of blood collected in buffered sodium oxalate (1%) anticoagulant was processed for DFM. The blood was centrifuged at 1000 x g for 15 min and plasma was placed on a clear grease free glass slide and applied a cover slip (18 mm square). This wet mount preparation was examined under low (10X) and high power (45X) objective of the dark field microscope. Utmost care was taken to examine as many microscopic fields as possible and a minimum of 100 high power fields were examined.

Urine

Five to six milliliter of urine samples collected with equal quantity of sterile PBS was immediately centrifuged at 3000 x g for 10 min. A drop of sediment was placed on a clear grease free glass slide, applied a cover slip (18 mm square) and was examined under low

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(10X) and high power (45X) objective of the dark field microscope and a minimum of 100 high power fields were examined

Polymerase chain reactions

Primers

The genus specific primers A and B designed by Merien *et al.* (1992) were obtained from Alpha DNA, Canada.

A: 5'-GGC GGC TCT TAA ACA TG-3'

B: 5'-TTC CCC CCA TTG AGC AAG ATT-3'

Preparation of template DNA from samples

In case of blood, one milliliter of blood was centrifuged at 1000 x g for 15 min and removed 0.5 ml of plasma. The plasma was further centrifuged at 13000 x g for 10 min at 4°C, washed the sediment twice with sterile PBS and then resuspended in 15 µl sterile triple glass distilled water. Finally the samples in Eppendorf tubes were placed on a boiling water bath for 10 min and immediately kept on ice for 30 min. Before setting of PCR the samples were thawed and centrifuged at 5000 x g for 10 min and the supernatant was used as template for PCR. For urine and serum samples the procedure was similar to the blood plasma. For processing the kidney tissues, a tiny portion of kidney cortex was triturated with one milliliter of sterile PBS. Rest of the procedure was same as for blood. The template DNA from samples was subjected to genus specific PCR using the primers A and B. Each reaction was done in a total volume of 25 ìl. For five such reactions, a mastermix was prepared to contain the following reagents

Reagents	Quantity
Triple distilled water	68 ìl
Magnesium Chloride (25 mM)	2.5 ìl
PCR buffer (10 X)	12.5 ìl
Primer A (20 pM/ ìl)	5 ìl
Primer B (20 pM/ ìl)	5 ìl
dNTPs	5 ìl
Taq polymerase (3units/ ìl)	2 ìl

Twenty microliter of mastermix was distributed to five tubes. To twenty microliter mastermix added five microliter template. Negative control without template to monitor contamination was also set. The PCR was carried out in an automated thermal cycler (Eppendorf Mastercycler, Germany).

The programme of amplification included a first cycle of Denaturation at 94°C for 3 min Annealing at 63°C for 1.5 min Extension at 72°C for 2 min followed by 28 cycles of Denaturation at 94°C for 1 min Annealing at 63°C for 1.5 min Extension at 72°C for 2 min and a final cycle of of Denaturation at 94°C for 1 min Annealing at 63°C for 1.5 min Extension at 72°C for 12 min. The amplified product was detected by Submarine Agarose gel electrophoresis (SAE) on one per cent agarose gel in TAE buffer (I X).

METHOD OF ISOLATION

Isolation of *Leptospira* was attempted from five urine samples and twenty five kidney tissues of rodents captured from the neighbouring farm premises. In addition to this, blood samples from two dogs positive by DFM were also subjected to isolation trials. Fletcher's Semisolid Medium (Hi Media) with5-Flourouracil (100 µg/ml) was used for isolation.

Screw capped tubes containing three to five milliliters of culture medium were inoculated with one to two drops of whole blood or a drop of the plasma with aseptic precautions (Cruickshank *et al.,* 1975). The inoculum was thoroughly mixed with medium and one milliliter of medium was transferred to a second tube and from this to a third tube.

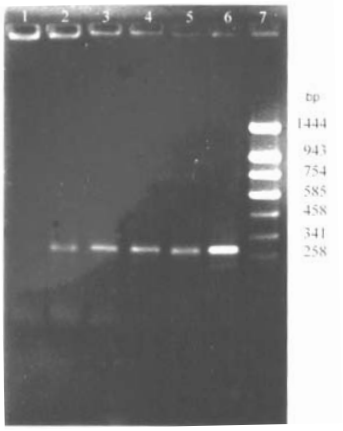
Isolation of leptospires from rodents was attempted by culturing the cortical portion of kidney and urine from the urinary bladder. Live rodents captured were anaesthetized with chloroform and opened with aseptic precautions. The tiny portion of kidney cortex collected by a single puncture with finely drawn sterile Pasteur pipette was inoculated to culture medium with aseptic precautions, mixed properly and then inoculated into two more tubes as described for blood. All the inoculated tubes were

incubated at 37°C for 24 h, followed by incubation at 28°C for two to three months. The tubes showing visual contamination were discarded. All the tubes were examined at weekly intervals by DFM and by culturing in blood agar. The contaminated tubes were discarded and those showing growth of leptospires were subcultured on to fresh media.

The urine samples from rodents were collected into sterile syringes and were cultured as for blood as described above.

RESULTS AND DISCUSSION

Polymerase chain reaction



Out of the 147 samples leptospiral DNA could be detected in nine samples.

Among the 117 clinical samples examined amplification of leptospiral DNA was observed only in eight serum samples, four from dogs and four from cattle. All these samples were long preserved ones received from District Veterinary Centre (DVC), Kottayam and were unsuitable for culturing. Of the 30 samples from rodents the kidney tissue of one bandicoot was positive by PCR

ISOLATION

Out of the 32 samples cultured for isolation seven samples were contaminated either with bacteria or fungus. Contamination was more common in urine samples and Fletcher's media with 5-FU were used mainly to culture urine samples. The bacterial contamination was evident from 24 h of incubation whereas the fungal contaminants took five to seven days to grow. Out of the 25 samples which were uncontaminated only one kidney sample from a bandicoot was positive for isolation of *Leptospira.* Rest of the samples was declared negative after incubation for a period of three months. The sample which was positive for isolation was positive by genus specific PCR and motile leptospires were demonstrable by 11th day of inoculation. The isolate was sub-cultured regularly at four weeks interval and the culture could be maintained in the laboratory for further identification.

Dark field microscopy (DFM)

Out of the 68 samples examined two blood samples were DFM positive and culturing was tried immediately. But later PCR and culture detected both the samples as negative for *Leptospira.* All the DFM negative samples were also negative by PCR and culture.Noubade *et al*. (2002) opined that PCR has the advantage that it does not require the isolation of the organisms and detected DNA from both viable and non-viable organisms. Isolation requires viable organisms to be present in the sample, which is again influenced by various factors such as method of collection, transportation and storage. Venkatesha and Ramadas (2001) could detect leptospires by PCR in more number of samples over culturing method and the possible reason could be that culturing needs viable organism where as PCR detects leptospiral DNA in samples. In the present study, though the results of DFM did not correlate with the results of PCR and isolation trials, DFM was found to be a reliable tool for demonstration of leptospires in cultures and also to check purity of cultures. In the present study PCR was found to be a rapid and reliable method for the diagnosis of leptospirosis.

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