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Evaluation of the polymerase chain reaction for early diagnosis of leptospirosis

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Summary. Early diagnosis of leptospirosis is important because severe leptospiral infection can run a fulminant course. The polymerase chain reaction (PCR) was evaluated for the detection of leptospires in clinical samples from patients with acute leptospiral infection. Blood and urine samples from 71 patients with leptospirosis were examined by PCR, culture or serology. Samples from 44 (62 %) patients with the diagnosis of leptospirosis were positive by PCR as compared to 34 (48 %) by culture. The presence of leptospires was demonstrated by PCR in 13 patients before the development of antibodies, as well as in two patients who were seronegative during their illness and at autopsy. Samples from 16 patients without leptospirosis were seronegative and culture negative, and also negative by PCR. We conclude that PCR is a rapid, sensitive and specific means of diagnosing leptospiral infection, especially during the first few days of the disease.

Introduction

Leptospirosis is considered to be one of the most widespread zoonoses worldwide.¹ Barbados, the most easterly of all the Caribbean islands, has a population of c. 260 000 inhabitants and a land mass of 430 km². The tropical climate, combined with the predominantly black soils which are slightly alkaline, and the dense population of rodents and dogs distributed throughout the country, make it an ideal environment for the maintenance and spread of leptospirosis.

In Barbados, leptospirosis has been recognised as an important disease affecting man since it was first described in 1939.² Since then, the disease has been investigated in hospital patients, dogs, livestock and wild-life, and leptospiral isolates have been identified.³ Until the end of 1989 only three serovars (*bim*, *copenhageni* and *arborea*), of the 20 or so detected in the Caribbean, were identified among over 100 *Leptospira* isolates from hospital patients in Barbados over the preceding 10-year period. A fourth serovar (*bajan*) has since been identified.³ Presently, the ratio of presumptive infecting serogroups in hospital patients is Autumnalis 60 %, Icterohaemorrhagiae 23 %, Ballum 14 % and others 3 %. On average, 33 cases of

severe human leptospirosis are recorded in Barbados each year with an associated case fatality rate of 14.2 %.³

Accurate diagnosis is important as it gives insight into the extent of the public health problem. Moreover, early diagnosis is particularly important for the clinical management of patients because treatment of leptospirosis can be effective if initiated early. Serology usually does not contribute to early diagnosis of leptospirosis, because antibodies become detectable around the seventh day of the disease. Conventional methods such as dark-field microscopy or culture to detect leptospires in clinical samples are either unreliable or too slow to contribute to a rapid diagnosis.^{1,4} Leptospires circulate in the blood of the patient until about the 10th day after the onset of symptoms.

With the introduction of PCR, rapid detection of small numbers of leptospires in clinical samples has become practical due to specific amplification of leptospiral DNA.^{5–7} This is important as leptospirosis can run a fulminant course and patients may die before the development of the characteristic clinical manifestations of leptospirosis or the appearance of leptospiral antibodies or both, and, therefore, the disease may go unrecognised. Post-mortem diagnosis may fail because leptospires may die before inoculation of culture medium and specific antibodies may not yet be

demonstrable in serum samples. In studies of genetic disorders⁸⁻¹⁰ and viral infections,¹¹⁻¹³ as well as for the detection of infectious agents that are difficult to cultivate or that do not grow in culture,¹⁴⁻¹⁶ PCR has been shown to be both sensitive and rapid. In this study, the PCR was applied to the sera and urine of hospital patients with leptospirosis to evaluate the detection of leptospiral DNA for the early diagnosis of leptospirosis.

Materials and methods

Clinical samples and culture

Blood and urine samples were obtained from 71 patients admitted to the Queen Elizabeth Hospital, Barbados, with a history and clinical manifestations of leptospirosis. Samples from 16 patients without leptospirosis were used as negative controls.

First blood (A1) samples from patients were collected on the day of admission, and a second (A2) sample was obtained no more than 5 days after the first. A third (convalescent) sample, if possible, was collected about 10-14 days later, for follow-up. The blood samples were allowed to clot at room temperature and the sera were frozen at -20°C for serology and PCR. Whole blood (A1 sample only) was cultured at the bedside in EMJH semi-solid medium,¹⁷ containing 5-fluorouracil $200\text{ }\mu\text{g/ml}$. Urine was collected from patients as soon after admission as possible, and was processed within 2 h of voiding. Samples (15 ml) of urine were centrifuged at $1500\text{ }g$ for 30 min and the pellet was resuspended in 1 ml of sterile phosphate-buffered saline (PBS), pH 7.2, and serially diluted for culture, or in $500\text{ }\mu\text{l}$ of PBS and frozen at -20°C for PCR. All cultures were examined routinely by dark-field microscopy for 26 weeks before the specimens were regarded as negative. Strains isolated from sera or urine samples were identified with monoclonal antibodies.¹⁸

Serology

Serum from the patients was examined by the *Leptospira* microscopic agglutination test (LMAT)¹⁹ with a battery of 22 serovars (used in the form of live culture antigens of a standard density) to establish seroconversion or a rise in titre. The enzyme-linked immunosorbent assay (ELISA)²⁰ was also performed to determine the IgM and IgG titres. Strain Patoc I (serovar *patoc*) was used as antigen in the ELISA. The diagnosis of leptospirosis was confirmed by a four-fold rise in titre or an initial titre of ≥ 800 in the LMAT with the appropriate multiple reactivity pattern, or an IgM titre of ≥ 160 in the ELISA, or a positive leptospiral culture (from blood or urine) or any combination of the three.

DNA isolation

DNA was extracted and purified from the serum and urine samples and concentrated on to diatoms in

the presence of guanidine thiocyanate (GuSCN).²¹ Briefly, $100\text{ }\mu\text{l}$ of serum or urine were added to $900\text{ }\mu\text{l}$ of L6 buffer (GuSCN 120 g, 0.1 M Tris-HCl, pH 6.4, 100 ml, 0.2 M EDTA 22 ml, Triton X-100 2.6 ml) with $40\text{ }\mu\text{l}$ of diatom suspension (diatoms 10 g, distilled water 50 ml, $500\text{ }\mu\text{l}$ of HCl 36% w/v). The mixture was vortex mixed, incubated at room temperature for 10 min and then centrifuged to pellet the diatoms complexed with DNA. After washing twice with L2 buffer (GuSCN 120 g, 0.1 M Tris-HCl 100 ml, pH 6.4), twice with ethanol 70% v/v, and once with acetone, the DNA-diatom complex was dried at 56°C for 10 min and the DNA was eluted in the presence of proteinase K $120\text{ }\mu\text{g/ml}$ solution at 56°C for 10 min. The proteinase K was subsequently inactivated by incubation at 100°C for 10 min.

PCR and detection

Primers (G1/G2 and B64-I/B64-II) for PCR have been described previously.⁷ The combined primer sets G1/G2 and B64-I/B64-II amplified DNA by PCR from all pathogenic *Leptospira* spp. DNA from strains of *L. interrogans*, *L. borgpetersenii*, *L. weilii*, *L. noguchii*, *L. santarosai* and *L. meyeri* strain ICF was amplified by G1 and G2, whereas that from strains of *L. kirschneri* was amplified by B64-I and B64-II. Amplification of DNA was carried out as described previously,⁷ with minor modifications. The reaction buffer consisted of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 3 mM MgCl_2 and Triton X-100 0.1%. Deoxyribonucleoside triphosphates (dATP, dCTP, dGTP and dTTP) were used at a final concentration of $250\text{ }\mu\text{M}$. For each reaction, 0.5 U of *Taq* DNA polymerase (Perkin-Elmer, Cetus) was added. PCR conditions consisted of 34 cycles of consecutive denaturation, annealing of primers and DNA chain extension (1.5 min at 94°C , 1 min at 55°C and 2 min at 72°C) preceded by an initial 5-min denaturation at 94°C and followed by a final elongation step at 72°C for 7 min. All PCR reactions were performed in a Pharmacia LKB Gene ATAQ controller (Pharmacia, Uppsala, Sweden). A blank control tube containing no added nucleic acids was run with every set of reaction mixtures to control for the inadvertent introduction of exogenous nucleic acids and appropriate positive controls were included in each run.

PCR amplification products were detected and identified as *Leptospira*-specific DNA by visualisation of the bands of the expected size on ethidium bromide-stained agarose gels and by Southern blot hybridisation with DIG-labelled oligonucleotide probes.²² A patient was scored positive if either the serum or urine sample gave a positive PCR result, i.e., a 285-bp fragment with primers G1/G2 or a 563-bp fragment with primers B64-I/B64-II, accompanied by corresponding hybridisation with the labelled probes.

The preparation of reaction mixtures, the DNA extraction (clinical samples and positive controls) and the subsequent amplification and detection of the PCR

products were all performed at different locations within one building. This strict spatial partition of the different technical steps involved in the PCR was necessary to prevent contamination. In addition, tables and equipment were decontaminated periodically with HCl 10%.

Results

Serology

Of the 71 patients with leptospirosis in this study, 69 (97%) were diagnosed serologically (table I); sera from the two other patients (both of whom died before a second sample could be taken) were seronegative, but yielded positive cultures. The mortality rate in this study was 16.9% (12 of 71). For the patients who were diagnosed by serology, 36 (50.7%) of the 71 A1 sera were positive and a further 32 (45.1%) A2 sera taken 1–5 days later were positive. IgM antibody titres were typically high with values of ≥ 1280 frequently being obtained from both A1 and A2 serum samples. One patient was diagnosed after serological analysis of autopsy specimens, and the two remaining patients died before the appearance of antibodies, but were positive by culture and by PCR.

Detection of leptospires by culture and PCR

Culture confirmed 34 (48%) of the 71 cases of leptospirosis. Serovars *bajan*, *bim* and *copenhageni* were isolated from one, 26 and seven patients, respectively. On average, cultures of blood and urine took 6 weeks (up to 13 and 10 weeks, respectively) for

Table I. Comparison of serology and culture for the detection of *Leptospira* spp. in the blood or urine of 71 patients with leptospirosis

Year	Number of patients	Number positive for leptospirosis by	
		serology	culture
1990	15	13*	12
1991	19	19	6
1992	14	14	10
1993	23	23	6
Total (%)	71 (100)	69 (97)	34 (48)

*Two patients were seronegative but culture positive.

Table II. Comparison of PCR and culture for the detection of leptospires in the blood of patients with leptospirosis

Blood culture result	PCR result		
	Positive	Negative	Total
Positive	21	12	33
Negative	20	9	29
Total	41	21	62

Table III. Comparison of PCR and culture for the detection of leptospires in the urine of patients with leptospirosis

Urine culture result	PCR result		
	Positive	Negative	Total
Positive	5	2	7
Negative	5	8	13
Total	10	10	20

leptospires to grow. Sera from 62 patients were available for PCR, of which 41 (66%) were positive with either G1/G2 or B64-I/B64-II primer sets (table II). Leptospiral culture was positive in 33 (53%) of these 62 patients. Urine samples were available from 20 patients, and of these, 10 (50%) were positive by PCR and seven (35%) were positive by culture (table III). When results from either blood or urine were combined, PCR detected 44 (62%) of the 71 cases and culture detected 34 (48%). PCR demonstrated the presence of leptospiral DNA in 20 (69%) of 29 sera and five (38%) of 13 urine samples from which no leptospires could be cultured, as well as in 21 (64%) of 33 sera and five (71%) of seven urine samples that yielded isolates identified as *bajan*, *bim* or *copenhageni* (tables II and III). Additionally, PCR demonstrated the presence of leptospires in 13 A1 serum samples that were seronegative; of these, only six were eventually culture positive.

All samples were tested independently two or more times by PCR and gave reproducible results, and DNA bands from PCR-positive samples were visible on Southern blots (figure). No DNA band was observed on gels or Southern blots for any of 16 negative control patients, or any blank sample.

Discussion

In the last 4–5 years, PCR technology has proven its value for the direct demonstration of fastidious pathogenic micro-organisms in clinical material.^{5–7, 11–16} The detection of leptospires by PCR at an early stage of infection can be of value for the diagnosis of leptospirosis when other methods fail or prove unreliable. This study showed the practical value of PCR for the early diagnosis of leptospirosis in patients infected by serovars prevalent in Barbados, especially before antibodies are detectable. PCR was more sensitive than culture for the detection of leptospires in both sera and urine samples from patients with severe leptospirosis. The positive results by PCR in seronegative samples demonstrated that the assay was able to detect leptospires in sera even before the development of antibodies. This most probably was indicative of the high sensitivity of PCR; false positive results were virtually excluded by use of correct controls and precautions. This study demonstrated that both PCR and culture were more often positive

Figure. Agarose gel electrophoresis (A) and Southern blot (B) analyses of PCR-amplified DNA extracted from serum and urine samples and amplified with primers G1/G2 (285-bp product) or B64-I/B64-II (563-bp product). Lane 1, mol. wt marker VIII (mixture of digests: pUC BM21·HpaII and pUC MB21·DraI + HindIII; Boehringer Mannheim); 2, blank (no DNA); 3, DNA from *copenhageni* amplified with G1/G2; 4, DNA from *bim* amplified with B64-I/B64-II; 5 and 6, DNA from samples amplified with G1/G2; 7–12, DNA from samples amplified with B64-I/B64-II. Southern blot hybridisation (at 55°C) with DIG-tailed oligonucleotide probes derived from the sequence generated by G1/G2-primed PCR or by B64-I/B64-II-primed PCR.²²

for sera than for urine samples. These results contrast with those of Bal *et al.*²² who found PCR analysis of urine samples to be more suitable for the detection of leptospiral DNA. In their study, a large volume of urine (up to 300 ml) was examined for increased sensitivity. The patients in this study were severely ill and usually oliguric, so it was frequently difficult to obtain urine for analysis. This will be the case if the infecting serovars cause severe illness accompanied by significant renal impairment.

Failure of leptospires to grow in culture was observed in this study and may be attributable, at least in part, to the fastidiousness of leptospires. Positive PCR results in culture-negative samples may indicate the amplification of DNA from non-viable leptospires. These samples frequently had high antibody titres, which could greatly reduce the possibility of isolating leptospires in culture. The high IgM titres observed in our patients underscored the significant interaction that occurs between the patients' immune system and the infecting leptospires.

PCR failed to detect leptospiral DNA in some samples from patients with leptospirosis. PCR may fail when there are inhibitory factors present in the samples that impede the amplification process,²¹ as well as when leptospires are present in very low numbers below the detection level of 1–10 lepto-

spires/ml.⁶ Similar levels of sensitivity for PCR have been reported for treponemes^{15,23,24} and borrelias.²⁵ The lower sensitivity of PCR and culture (relative to serology) may be attributed to the lower number of leptospires in the blood or urine of patients with less severe infections. Positive cultures were usually obtained in the more severe infections, i.e., those involving *copenhageni* or *bim*, which are more widespread in their distribution than are other serovars that occur in Barbados. The severity of infection affects the time (and stage of illness) when patients present with symptoms: the more severe the infection, the earlier the stage at which patients present at hospital.

The principal value of the PCR lies in the ability to obtain a definitive diagnosis during the acute stage of the illness, before antibodies are detectable, while treatment may be effective, and several weeks before culture results are available. This study showed that PCR can be used to detect leptospires in the acute stage of leptospiral infections. We believe that PCR will be a valuable tool for early diagnosis of leptospirosis.

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