DIAGNOSIS OF SCRUB TYPHUS IN MALAYSIAN ABORIGINES USING NESTED POLYMERASE CHAIN REACTION

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Abstract. A rapid diagnostic system for scrub typhus using nested polymerase chain reaction (PCR) was applied to clinical samples from Malaysian Aborigines. Whole blood from twenty-four patients suspected of scrub typhus infection were tested using nested polymerase chain reaction and sera were evaluated by the indirect immunoperoxidase test. Antibody responses towards *Rickettsia tsutsugamushi* were observed in seventeen patients with the majority having high titers of IgG antibodies. Seven patients were seronegative. The nested PCR amplified *R. tsutsugamushi* DNA from six patients, of which two were negative serologically and four had high titers of IgG antibodies. Second samples collected seven days after treatment were negative by PCR testing. Nested PCR is highly sensitive and specific and may be used to provide rapid confirmation of scrub typhus cases in endemic region.

INTRODUCTION

Scrub typhus is a major cause of febrile illness in the Asia Pacific region (Rapmund, 1984). The infection is caused by *Rickettsia tsutsugamushi* which is transmitted to humans through the bites of Leptotrombidium mite. Classical clinical manifestations of this infection are often absent and mimic other tropical infections such as typhoid fever, leptospirosis and dengue (Shirai, 1978). The common laboratory diagnostic methods are immunological techniques using Weil Felix, indirect immunofluorescence and indirect immunoperoxidase tests. However, serological tests are retrospective and reinfection may occur even after the development of specific immunity against *R. tsutsugamushi* (Groves and Kelly, 1989). In addition, the heterogeneity of *R. tsutsugamushi* in an endemic region may reduce the sensitivity of the serological test as limited local strains are available for diagnostic purposes. The amplification of a conserved region of genomic DNA by the polymerase chain reaction (PCR) has proved to be a valuable tool in the diagnosis of various typhus infections including scrub typhus (Furuya *et al*, 1991; Sugita *et al*, 1992; Furuya *et al*, 1993).

A high prevalence of antibody to *R. tsutsugamushi* has been demonstrated among Malaysian Aborigines (Cadigan *et al*, 1972; Brown *et al*, 1978) but confirmation of scrub typhus is always hampered due to the non-specificity of the symptomatology in patients and lack of suitable laboratory tests. In this study, we describe our experience of using PCR for detection of *R. tsutsugamushi* in this group of patients.

MATERIALS AND METHODS

Bacterial strains

Control DNA preparations were prepared as described by Furuya *et al* (1991) from *R. tsutsugamushi* prototypes (Gilliam, Karp, Kato serotypes), tick-borne rickettsia, "TT118" and *R. typhi* Wilmington which were cultured in fertile chicken eggs (Specific Pathogen Free Avian Supply). Briefly, rickettsial suspensions were mixed with sodium dodecyl sulphate at 4°C overnight before incubated with 3x cystallized chicken egg white lysozyme (Sigma) at a final concentration of 2 mg/ml for 30 minutes in ice. The lysate was then incubated with Proteinase K at a final concentration of 0.2 mg/ml for 1 hour at 55°C. This was followed by phenol-chloroform extraction and ethanol precipitation of the DNA. Purified bacterial DNA was then resuspended in 20 µl of distilled water and used as DNA template. Uninfected egg yolk sac was used as negative control.

Clinical samples

The patients investigated were twenty-four Malysian Aborigines (aged from 5 months to 84 years) admitted to a district hospital from March to

May 1995. They were having fever and suspected of scrub typhus at the time of investigation. Whole blood samples were collected in EDTA blood collection tubes for PCR testing and sera were obtained for serology evaluation using the indirect immunoperoxidase test. Minocyclines was administered to all patients after admission. Second whole blood samples were collected seven days after treatment and subjected to PCR testing.

Nested polymerase chain reaction

PCR was performed as described by Furuya *et al* (1993) with slight modification. The PCR amplification mixture (total volume, 50 µl) contained 1.5 mM $MgCl_2$; 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 0.001% (w/v) gelatin; 200 µM each dATP, dGTP, dCTP and dTTP; 0.2 µM primers 34 and 55, 1.25 U of *Taq* polymerase (Boehringer Mannheim) and 5 µl of template DNA. The mixture was denatured at 94°C for 30 seconds, annealed at 57°C for 2 minutes and then chain was extended at 70°C for 10 minutes in a thermal cycler for 30 cycles. First PCR product was diluted 1:10 before used for second PCR using primers 10 and 11. The amplification products was electrophoresed on 1.5% agarose gels which were then stained with ethidium bromide and observed under ultraviolet transillumination. When the 483 bp - specific band was detectable, the sample was designated positive.

Indirect immunoperoxidase test

Patients' sera were tested for the presence of IgG and IgM towards mixtures of *R. tsutsugamushi* prototypes (Gilliam, Karp and Kato) as described by Kelly *et al* (1990).

RESULTS

The PCR assay results for control strains of *R. tsutsugamushi* are shown in Fig 1. The first PCR product generated by the primers 35 and 55 was 1003 bp whereas the second PCR product using primers 10 and 11 was 483 bp. These were observed for all the *R. tsutsugamushi* prototypes but not for TT118, *R. typhi* and uninfected egg yolk sacs. Of the 24 whole blood samples tested, six were positive by PCR (Fig 2).

The indirect immunoperoxidase test revealed the presence of antibody responses in 17 of 24

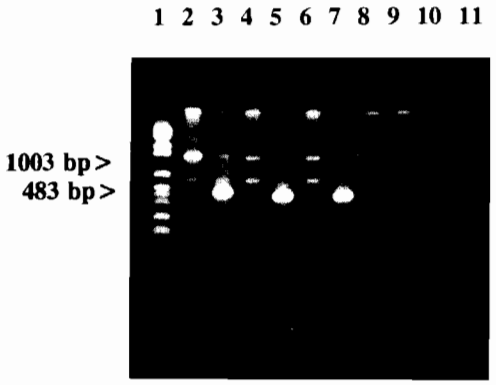

Fig 1–Analysis of PCR amplification products on agarose 1.5% w/v gels. PCR products amplified from SPAFAS chicken yolk sacs infected with rickettsiae. *R. tsutsugamushi* serotype Gilliam (lanes 2 and 3), Karp (lanes 4 and 5), Kato (lanes 6 and 7), *R. typhi* (lanes 8 and 9), TT118 (lanes 10 and 11). PCR products were obtained with primers 34 and 55 (lane 2, 4, 6, 8, 10) and primers 10 and 11 (lanes 3, 5, 7, 9, 11). DNA molecular weight marker VI, Boehringer Mannheim (lane 1).

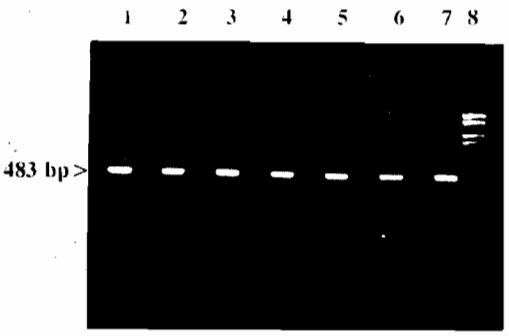

Fig 2–Analysis of second PCR product amplified from whole blood samples obtained from 6 patients suffering from scrub typhus. Control *R. tsutsugamushi* serotype Gilliam (lane 1), DNA extracted from whole blood samples of 6 febrile patients suspected of scrub typhus (lanes 2, 3, 4, 5, 6,7), DNA molecular weight marker VI, Boehringer Mannheim (lane 8).

patients tested (Table 1). High antibody titers (IgG ≥ 1:1600) were detected in 15 patients. Seven patients were seronegative. Two patients with seronegative result (IgG and IgM < 1:50) and 4 with high titers of IgG but undetectable IgM (IgG ≥ 1:1600, IgM < 1:50) were positive for scrub typhus by nested PCR. No amplification of rickettsial DNA

Table 1

Laboratory diagnosis of scrub typhus by nested PCR and the indirect immunoperoxidase test.

Antibody titers		No. patients	No. PCR positive
IgG	IgM		
≤ 1 : 400	< 1 : 50	2	0
≥ 1 : 1,600	> 1 : 50	3	0
≥ 1 : 1,600	< 1 : 50	12	4
< 1 : 50	< 1 : 50	7	2
Total		24	6

was detected from the second whole blood samples. Seroconversion was not observed.

DISCUSSION

The patients described in this study were Aborigines who stayed in Orang Asli settlements in the jungle or fringes of villages. Due to their life style and association with agricultural activities, they were subjected to very high risk of scrub typhus infection (Brown *et al*, 1978). Cadigan *et al* (1972) found a prevalence of 73% in adult Aborigines from "deep jungle", 48% from "fringe" areas, and 8% from kampongs (villages); Robinson *et al* (1976) reported prevalence rates of 6-69% in groups of rural Malaysia. Although systemic investigation of febrile patients revealed a high incidence of scrub typhus among indigenous populations, the reported incidence of scrub typhus had been low (Brown *et al*, 1978). These could be due to the variability of disease manifestations, inapparent infections caused by weakly pathogenic strains and lack of laboratory tests for confirmation.

High seroprevalence was also observed in this study where 17 (71%) of the 24 patients' sera were reactive to *R. tsutsugamushi* and only 7 were negative. With indirect immunoperoxidase technique, high IgG antibody titers with no IgM detected indicates the persistence of IgG antibody after a primary infection, whereas the absence of both IgG and IgM suggests no exposure to *R. tsutsugamushi* or a very early stage of infection. It has been reported that these antibodies persist for six to ten years and may even longer after reinfection (Bozeman and Elisberg, 1967; Shishido, 1962). In active case of scrub typhus, no or very low antibody titers is detected if proper antibiotic therapy is initiated early in the course of the disease (Smadel *et al*, 1954). Interpretation of antibody titers therefore requires a certain amount of judgement based on the history of the individual patient and the characteristics of the patient population (Strickman, 1994). For patients from endemic regions where reinfection and subclinical infections are not uncommon, antibody tests may fail to identify rickettsial infection early enough to affect the management.

In this study seroconversion was not observed in the second serum samples collected seven days later. We speculate that in this highly endemic area persistence of the organism following infections may be common and recrudescence of dormant infections might result in a lesser rise in antibody titer. Treatment given promptly after infections might inhibit the rise of antibodies.

The gold standard for the confirmation of scrub typhus infection is the isolation of organisms from patients. This however requires animal facilities, is laborious and time consuming. Confirmatory diagnosis of scrub typhus made serologically is long and only achieved after the acute illness has resolved. A rapid, sensitive and specific test provided by PCR technologies which detects the presence of bacterial genomes in the clinical specimens, offers an alternative for the diagnosis and confirmation of the infections.

The nucleotide sequence of the primers used in this study were based on the established nucleotide sequence of a mature 56 kDa protein in serotype Gilliam of *R. tsutsugamushi* (Ohashi *et al*, 1992). In our study, PCR was successfully used to attempt specific DNA amplification from control strains of *R. tsutsugamushi* prototypes and six clinical samples obtained from patients with scrub typhus. The amplification of a conserved DNA region is of particular useful in view of the existence of multiple antigenic types of *R. tsutsugamushi* in this region (Shirai *et al*, 1979).

Several reports showed that PCR revealed rickettsial infection during the acute rickettsemia phase, which occurs before the antibody could be detected (Furuya *et al*, 1991; Furuya *et al*, 1993). Thus diagnosis is possible in the early stage of the illness. In our study, The amplification of Rickett-

sial DNA from two patients with sera negative towards *R. tsutsugamushi* may suggest an early infection. Four patients who had high titers of IgG antibodies might have reinfected either by the similar or other antigenic types of *R. tsutsugamushi*.

The PCR did not amplified DNA from blood obtained from patients who had treated successfully with antibiotics. At this time, *R. tsutsugamushi* might have been eradicated from patients' blood. Although the precise number of rickettsiae found in whole blood during acute infection in humans is not known, it has been reported that the nested PCR enabled the detection of two copies of *R. tsutsugamushi* DNA in the specimen (Yoshida *et al*, 1994).

Nested PCR is therefore a promising test for scrub typhus as it is rapid, highly sensitive and specific and it may provide rapid confirmation of scrub typhus cases in endemic region. This technique may sought for the disparity between the low reported incidence of scrub typhus and the high prevalence of antibody to *R. tsutsugamushi* in indigenous populations.

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