

ISOLATION AND PCR DETECTION OF RICKETTSIAE FROM CLINICAL AND RODENT SAMPLES IN MALAYSIA

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Abstract. Isolation of rickettsiae from patients' blood samples and organ samples of wild rodents from areas with high seroprevalence of rickettsial infections was attempted using cell culture assay and animal passages. L929 mouse fibroblast cells grown in 24 well tissue culture plate were inoculated with buffy coat of febrile patients and examined for the growth of rickettsiae by Giemsa, Gimenez staining and direct immunofluorescence assay. No rickettsiae were isolated from 48 patients' blood samples. No symptomatic infections were noted in mice or guinea pigs infected with 50 organ samples of wild rodents. There was no rickettsial DNA amplified from these samples using various PCR detection systems for *Orientia tsutsugamushi*, typhus and spotted fever group rickettsiae.

INTRODUCTION

Rickettsioses (scrub typhus, murine typhus and tick typhus) constitute a very significant, but often unrecognized fraction of acute febrile diseases burdening many populations, especially in developing countries. Several seroprevalent studies in the Southeast Asian regions have shown the increasing importance of rickettsial infections (Rapmund, 1984; Rosenburg, 1997). In Malaysia, several studies (Ho *et al*, 1997; Tay *et al*, 1999; 2000) suggested that rickettsial infections are endemic in this country, with scrub typhus continuing to be a public health problem. Tick and murine typhus are apparently much more widespread than scrub typhus in this country. However, despite high seroprevalence of rickettsial infections, there had not been any recent isolates.

Isolation of rickettsiae is a prerequisite for the characterization of a new species and for the delineation of new rickettsial diseases. The isolation of rickettsiae has previously been accomplished by the inoculation of clinical samples into embryonated hen eggs and laboratory animals. During recent years, the devel-

opment of cell culture systems for viral isolation has led to an increase in the isolation of rickettsiae (Raoult and Roux, 1997). The early diagnosis of scrub typhus is now possible in the early stage of the illness using polymerase chain reaction (PCR) (Furuya *et al*, 1993). By amplification of the gene encoding the 56 kDa antigen of *Orientia tsutsugamushi* (OT) in blood (Furuya *et al*, 1993) and in tissues (Sugita *et al*, 1992), PCR revealed rickettsial infection during acute rickettsemia phase, which occurs before the antibody increases. Similarly, for typhus group (TG) and spotted fever group (SFG) rickettsial infections, PCR provides a means for the rapid detection of rickettsial DNA in clinical samples (Carl *et al*, 1990; Williams *et al*, 1994).

The objective of this study was to attempt isolation of various species of rickettsiae from patients' blood and rodent samples, collected from areas with high antibody prevalence of human rickettsial infections.

MATERIALS AND METHODS

Clinical samples

These were 48 whole blood samples

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collected from febrile patients attending Slim River Health Center, Malaysia in December, 1997. A total of 5 ml of blood was drawn from each patient, of which 1 ml was placed in an eppendorf tube for serum collection and 4 ml was placed in EDTA collection tubes. The blood was allowed to settle for at least 2 hours before 1 ml of the buffy coat was collected. Samples were kept in liquid nitrogen prior to use.

Rodent samples

These were 50 organ tissues (livers, spleens and kidneys) of wild rodents caught near the Selangau Health Center, Sarawak (Tay *et al*, 1998). The kidney, liver and spleen of each rodent were pooled and homogenized in Snyder's I diluent, pH 7.4 to a 20% (w/v) suspension using a glass homogenizer immersed in ice. Each sample was aliquoted in 1 ml volume and kept at -70°C before use.

Rickettsiae

The reference rickettsial strains were cultured in embryonated hen eggs and maintained in the laboratory of the Institute for Medical Research, Kuala Lumpur: OT (Karp strain), a TG rickettsiae (*Rickettsia typhi*, RT) and a SFG rickettsiae, TT118 strain.

Isolation of rickettsiae using cell culture assay

The isolation of rickettsiae from patients' blood samples was performed using cell culture assay. The assay was not applied to the organ samples of rodents due to the high rate of bacterial contamination when these samples were used. A total of 2×10^6 L929 mouse fibroblast cells (ATCC CCL-1) were seeded in each well of a 24-well tissue culture plate (Costar, USA) and incubated for 3 days to obtain a confluent monolayer. A volume of 300 μ l of buffy coat extracted from patients' blood sample was then added into 3 wells. The tissue culture plate was centrifuged at 700g at 37°C for 1 hour. The inoculum was then discarded and fresh medium (RPMI 1640 medium supplemented with 2% heat-inactivated fetal calf serum) was added. The plate was then incu-

bated at 37°C for 10-14 days with changes of fresh media every 3-4 days.

For detection of the presence of rickettsiae, infected cells were dislodged from the wells and centrifuged at 1,000g for 10 minutes in an eppendorf tube. The cells were then resuspended in 0.1 ml of PBS before being dotted onto plain slides, fixed and subjected to Giemsa and Gimenez staining. Another slide was fixed in acetone for 10 minutes at 4°C for direct immunofluorescence assay (DFA). DFA was performed using FITC-labeled antisera against OT, RT and SFG rickettsiae (provided by Naval Medical Research Institute, Bethesda, MD, USA). A volume of 5 μ l of the FITC-labeled antibody was added to each well dotted with infected cells. The slides were incubated in a moist chamber at 37°C for 30 minutes and washed twice with PBS. The slides were then soaked in 0.05% Erichrome B (INDX, USA) for not more than 5 minutes before washing twice in PBS. The slides were then air-dried before mounting with glycerine mounting medium. The slides were examined immediately at a 400x magnification with an ultraviolet microscope. Positive controls using egg-passaged rickettsiae (OT, RT and TT118 SFG rickettsiae) were included in each examination.

Isolation of rickettsiae using animal passage

This method was applied for isolation of rickettsiae from organ samples of wild rodents. Animals (ICR outbred mice and guinea pigs) were supplied by the Animal Resource Unit of the Institute for Medical Research, Kuala Lumpur, Malaysia. For each homogenized sample, 0.5 ml was inoculated intraperitoneally into 2 adult male guinea pigs and another 0.2 ml was inoculated intraperitoneally into two 6-8 week-old ICR outbred mice. Animals were observed daily for any clinical signs and symptoms. At day 10, organs from mice (kidney, liver and spleen) were harvested and homogenized for a second passage. Blind passages were carried out after day 10 if no illness was observed in these animals. A sample was considered as negative for isolation of

rickettsiae after three passages. Guinea pigs were examined for the presence of antibody to OT, RT and TT118 SFG rickettsiae prior to and after 21 days of infection using indirect immunofluorescence assay (IFA).

Laboratory strains of rickettsiae, which included OT (Karp strain), RT and SFG rickettsiae (TT118 strain) were used as control organisms in this study. Briefly, 0.5 ml of RT and TT118-infected yolk sac suspension was inoculated intraperitoneally into 2 adult male guinea pigs and similarly 0.2 ml of Karp-infected yolk sac suspension was inoculated intraperitoneally into 6-8 weeks old ICR mice. Animals were observed daily for any clinical signs and symptoms. Sera from guinea pigs were collected by cardiac puncture prior to infection and 21 days after infection. Rickettsial antibodies against OT, RT and TT118 SFG rickettsiae were determined by IFA using rickettsial antigen, *ie*, OT, RT and TT118 SFG rickettsiae.

Indirect immunofluorescence assay (IFA)

Briefly, two fold dilutions of each serum with a starting dilution of 1:20 were added to individual antigen spots and incubated in a humidified chamber at 37°C for 30 minutes. After washing thrice in PBS for 5 minutes, 1:10 diluted fluorescein-conjugated anti-guinea pig antibody (Kirkegaard and Perry Laboratories, USA) was added to each antigen dot. Slides were then incubated at 37°C for 30 minutes, washed and air-dried before soaking in 1:40 dilution of freshly prepared Erichrome B (INDX, USA) for 2 minutes. The slides were rinsed several times in distilled water and air dried before mounting with 90% (v/v) glycerine mounting medium. The slides were then examined with an ultraviolet fluorescent microscope. Sera having titers of \geq 1:40 were considered positive.

Indirect immunoperoxidase (IIP) assay

To determine the presence of rickettsial antibody in the patients' blood samples from Slim River Health Center, IIP assay was performed as described by Kelly *et al* (1988).

Preparation of DNA template for PCR

DNA was extracted from 0.5 ml of samples using a phenol-chloroform extraction method as described by Furuya *et al* (1993). These samples were mixed with 1/10 volume of 10% sodium dodecyl sulfate (SDS) (final concentration of SDS, 1%) and incubated at 4°C for 16 hours. This was followed by the addition of 1/10 volume of a 10-fold concentration of TE buffer and 3x crystallized chicken egg white lysozyme (Sigma) (final concentration of 2 mg/ml) for 30 minutes in an ice bath. Proteinase K at a final concentration of 0.2 mg/ml was then added and the mixture were incubated at 55°C for 1 hour. The DNA in this lysate was purified by three extractions with an equal volume of a phenol-chloroform (1:1) mixture, followed by precipitation with 2 volumes of ethanol and resuspended in 20 μ l of distilled water. Five microliters of DNA sample was used in each run of PCR.

PCR conditions for detection of OT

Nested PCR was performed as described by Furuya *et al* (1993). The PCR amplification mixture (total volume, 50 μ l) contained 1.5 mM MgCl₂; 50 mM KCl; 10 mM HCl (pH 8.3); 0.001% (w/v) gelatin, 200 μ M each dATP, dGTP, dCTP and dTTP; 0.2 μ M primers (p34 and p55), 1.25 U of *Taq* polymerase (Promega, USA) and 5 μ l of template DNA. The mixture was denatured at 94°C for 30 seconds, annealed at 57°C for 2 minutes and at 70°C for 2 minutes for chain reaction. This was run for 30 cycles and extended at 70°C for 10 minutes in a thermal cycler (ThermojeT, Belgium). The first PCR product was then diluted 1:10 for the second PCR amplification using internal primers, p10 and p11. The amplification product was electrophoresed on 1.5% agarose gel which was then stained with 0.5 μ g/ml of ethidium bromide solution and observed under ultraviolet transillumination. When the 500 bp-specific band was detectable, the sample was considered as positive. OT reference strains, Karp in egg yolk suspension, was used as a positive control strain whereas uninfected egg yolk suspension was used as a negative control.

PCR conditions for detection of TG and SFG rickettsiae

PCR amplifications were performed by using oligonucleotide primer pairs, RpCS.877p and RpCS.1258n generated from the citrate synthase gene of *R. prowazekii* and primer pairs, Rr190.70p and Rr190.602n generated from the 190 kDa antigen gene of *R. rickettsii* (Regnery *et al.*, 1991). Briefly, a total of 100 µl of the reaction mixture, which contained 5 µl of prepared sample, 66.5 µl of distilled water, 10 µl of 10x *Taq* buffer (Promega, USA), 10 µl of deoxynucleotide triphosphate (10 mM dNTPs) in distilled water, 5 µl of each component of the primer pair, and 0.5 µl of *Taq* polymerase (Promega, USA) was prepared and processed by using a thermal cycler (ThermojeT, Belgium). The PCR was run for 35 cycles of amplification that consisted of denaturation at 95°C for 20 seconds, annealing at 48°C for 30 seconds, and sequence extension at 60°C for 2 minutes, according to the protocol described by Regnery *et al.* (1991). To verify the result of the PCR amplification, 10 µl of the amplified material was electrophoresed at 100 V in a 1% agarose gel for 1 hour. The detection of a PCR product of 381 bp was considered as positive for TG rickettsiae whereas the detection of a PCR product of 532 bp was considered as positive for SFG rickettsiae.

RESULTS

Serological findings of febrile patients

Of the 48 patients attending Slim River Health Center, 32 were males and 16 were females (Table 1). All were febrile at the time of diagnosis, 41 (85.4%) had headache and 31 (64.6%) had pharyngitis. None has eschar and lymphadenopathy. The mean \pm SD age was 38.75 \pm 18.13 years (range: 12-73 years). Most of the patients were Indians (60.4%) and Malays (31.3%). Eight (16.7%) patients had IgG antibodies to OT and 10 (20.8%) patients had IgG towards SFG rickettsiae. IgM antibodies to OT, RT and SFG rickettsiae were detected in 16 (33.3%), 1 (2.1%) and 37 (77.1%) of

the febrile patients. The highest geometric mean titer (474.4) was observed with IgG antibodies to OT. The overall serological results of these patients showed that rickettsial antibodies with IIP titers of \geq 1:50, either IgG and/or IgM, were detected in 44 (91.7%) patients. SFG rickettsial antibodies were most prevalent (83.3%), followed by antibodies to OT (25.0%) and RT (2.1%). Thirteen patients were bled for the second time after two weeks. They were healthy at the time of blood collection. Seroconversion was only noted for SFG rickettsial antibodies in two patients (data not shown).

Isolation of rickettsiae using cell culture assay

Monolayers of infected L929 cells were disintegrated after incubation of 10-14 days. No rickettsiae could be identified from Giemsa- or Gimenez-stained smears or by DFA assay. No rickettsiae were isolated from the 48 patients' blood samples.

Isolation of rickettsiae using animal passage

Mice infected with OT (Karp strain) appeared sick on day 7-10 with ruffled fur, cold and inactive. The mice eventually died on day 14. Guinea pigs infected with either RT or SFG rickettsiae appeared sick on day 4 with ruffled fur, fever, losing weight and inactive. Ear inflammation as the result of vasculitis, rashes on the footpad and scrotal area were observed as compared to the uninfected guinea pig. Scrotal swelling was observed on day 3 and this led to rashes and hemorrhage on the following two days. Necrosis occurred eventually on ears and scrotal areas and guinea pigs died on the eighth day post-infection. High antibody titers ($>$ 1:6,400) were obtained from the surviving guinea pigs on day 21.

All the mice and guinea pigs inoculated with organ tissues of wild rodents were asymptomatic at the end of experiment. Upon dissection, no splenomegaly and ascitis accumulation was observed in the mice. Antibody against OT, RT and SFG rickettsiae was not detected in guinea pigs inoculated with these samples after 21 days.

Table 1
Demographic and serological findings of febrile patients attending Slim River Health Center.

	No. of patients	No. of patients seroreactive to		
		OT	RT	SFGR
Sex				
Male	32	9	1	27
Female	16	3	0	13
Total	48	12(25.0%)	1(2.1%)	40(83.3%)
Age groups (years)				
<21	11	1	0	11
21-30	6	1	0	4
31-40	12	2	0	10
41-50	10	3	0	6
>51	9	5	1	9
Total	48	12(25.0%)	1(2.1%)	40(83.3%)
Race				
Malay	15	6	1	18
Indian	29	4	0	19
Others	4	2	0	3
Total	48	12(25.0%)	1(2.1%)	40(83.3%)
Antibody titer (class)				
1:50 (G)		1	0	3
1:50 (M)		6	1	6
1:100 (G)		1	0	0
1:100 (M)		0	0	9
1:200 (G)		1	0	1
1:200 (M)		9	0	10
1:400 (G)		0	0	2
1:400 (M)		1	0	7
1:800 (G)		2	0	4
1:800 (M)		0	0	3
≥1:1,600 (G)		3	0	0
≥1:1,600 (M)		0	0	2
Reciprocal geometric mean titer				
IgG		474.4	0	263.2
IgM		76.9	49.9	192.1

OT: *O. tsutsugamushi*; RT: *R. typhi*; SFGR: spotted fever group rickettsiae, TT118.

PCR detection of OT, TG and SFG rickettsiae

No positive amplification was obtained with the patients' blood samples or organ samples of wild rodents using PCR detection systems for OT, TG and SFG rickettsiae despite positive findings with samples simulated with DNA of rickettsiae.

DISCUSSION

Patients attending the Slim River Health Center were selected in this study based on the fact that high seroprevalence of antibody to rickettsiae was reported among those febrile patients attending the health center and therefore higher probability of isolating rickettsiae

from these patients. The Slim River Health Center cares for patients who live and work mainly in rural areas. Most of the villagers were working in the agricultural sector, either as rubber tappers, farmers or oil palm plantation workers. In a previous study (Tay *et al*, 2000), it was reported that of 259 febrile subjects attending the Slim River Health Center, 189 (73.0%) were seropositive to at least one rickettsial group, of which the seroprevalence of OT, RT and SFG rickettsiae among these patients were 32.0%, 40.5% and 59.5%, respectively. The clinical presentations of these patients were mild and there were no distinct clinical signs and symptoms of rickettsial infections, such as eschar, lymphadenopathy or rashes. In this study, despite the serological evidence of rickettsial infections, the causative agents for the febrile illness were not isolated.

Several methods for rickettsial isolation and detection such as centrifugation-shell vial technique (Marrero and Raoult, 1989), genomic amplification of rickettsial DNA by PCR (Carl *et al*, 1990; Sugita *et al*, 1992; Furuya *et al*, 1993; Williams *et al*, 1994), direct demonstration of rickettsiae in skin biopsy specimens by using DFA and IFA of rickettsiae in circulating endothelial cells (Drancourt *et al*, 1992) have greatly enhanced the detection and diagnosis of rickettsiae. In this study, L929 cells grown in a 24-well tissue culture plate were used for inoculation of patients' blood samples. A smaller surface area of the tissue culture well would enhance the ratio of the numbers of rickettsiae to the number of cells and might allow better isolation of rickettsiae. Buffy coat of febrile patients was used as inoculum in this study for isolation of rickettsiae as higher numbers of endothelial cells are usually present in the buffy coat as compared to whole blood sample. Endothelial cells are the primary targets for rickettsial infections and this has been supported by histopathologic observations of biopsy specimens and necrotoxic tissues from patients with spotted fever (Greene *et al*, 1978) and of experimental infections of cultured human endothelial cells (Silverman and Bond, 1984). Low speed centrifugation was performed after inoculation of samples in this study to enhance

rickettsiae attachment to and penetration of the cells (Kelly *et al*, 1991).

No rickettsiae were isolated from the patients' blood samples in this study and the causes of the febrile illness suffered by these patients remained unknown. The negative isolation of rickettsiae from the patients' samples could be due to the absence or low numbers of rickettsiae in these samples. The difficulty in the isolation of rickettsiae had been documented (Burgdorfer *et al*, 1979; Kawamura *et al*, 1980). The failure to isolate rickettsiae from the blood of about 55% of patients later proven serologically to have had scrub typhus had been reported (Brown *et al*, 1983; Chouriyagune *et al*, 1992). The sensitivity and specificity of PCR systems used in this study confirmed the negative isolation of OT, TG and SFG rickettsiae from the samples.

Most patients (91.7%) in this study had antibody against at least one type of rickettsial group (Table 1); however, the presence of antibodies in these patients may not always indicate active/current infection. In addition acute rickettsemia may occur in a very short time, and as most of the patients had antibody detected this may provide protection to the patients against rickettsemia which had occurred earlier. This could be an explanation for the absence of rickettsiae at the time of blood collection.

In this study, direct inoculations of organ tissues of wild rodents into cell culture have resulted in bacterial contamination with most samples. Suitable laboratory animals were therefore used for isolation of rickettsiae from these samples. There was no successful isolation of rickettsiae from organ tissues of wild rodents in this study. Samples passaged three times in the mice were negative for OT infection whereas none of the guinea pigs had any signs and symptoms as demonstrated by the laboratory strains of RT or TT118 SFG rickettsiae. This finding was similar to those of Marchette (1966) who reported no febrile period or scrotal reaction in guinea pig infected with tick or rodent tissues from this country.

This suggests that the local strains of rickettsiae are probably of low virulence or present in a very low number. The presence of rickettsial antibodies in majority of wild rodents (Tay *et al*, 1998) may also provide protection to the rodents by clearing off the rickettsiae from blood circulation in the early stages of infection. Further studies are necessary to study the progression of these infections in human and rodents in order to improve the isolation of the rickettsiae.

ACKNOWLEDGEMENTS

This study was funded by the Intensification of Research Priority Areas Program grant No. 06-05-01-0071 and 06-02-03-0302.

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