EARLY DIAGNOSIS OF SCRUB TYPHUS IN THAILAND FROM CLINICAL SPECIMENS BY NESTED POLYMERASE CHAIN REACTION

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Abstract. The early detection of scrub typhus in Thailand by nested polymerase chain reaction (PCR) is presented. The diagnosis of scrub typhus, from clinical samples obtained from hospitals in the northern part of Thailand, by nested PCR was compared to immunofluorescence (IF) and Weil-Felix (WF) tests. The primer pairs used for the nested PCR were designed on the basis of the nucleotide sequence of the gene that encodes the 56-kDa antigen, and RFLP analysis was used for identification. Clotted blood from 80 patients suspected of scrub typhus infection were tested. With the IF test, antibodies for *Orientia tsutsugamushi* were observed in 38 patients checking IgM and IgG titers. Only 21 patients showed positive seroconversion while 17 patients were negative. For the WF test, only 13 patients gave a positive seroconversion. In the early stage of infection, 19, 13 and 3 patients were detected with a sensitivity of 90.47% (19/21), 61.90% (13/21) and 14.28% (3/21) by the nested PCR, IF and WF test respectively. Two patients who were negative for seroconvesion by IF and WF were positive by nested PCR. Therefore, this suggests that nested PCR is applicable for specific rapid diagnosis at an early stage of scrub typhus in endemic regions.

INTRODUCTION

Amplification of genomic DNA by polymerase chain reaction (PCR) has proven to be a valuable tool in the diagnosis of some infectious diseases, especially those caused by organisms which are present in minute quantities or are either too dangerous or difficult to isolate (Fredricks and Relman, 1999). *Orientia (*formerly *Rickettsia) tsutsugamushi* (Tamura *et al*, 1995), the causative agent of scrub typhus fever, is transmitted to humans through the bite of an infected Leptotrombidium mite. Scrub typhus accounts for up to 23% of all febrile episodes in endemic areas of the Asia-Pacific region (Brown *et al*, 1976). Clinical manifestation of this infection often mimics other tropical infections and can not be easily differentiated, thus it is frequently called pyrexia of unknown origin (PUO) (Silpapojakul, 1997; 1999). The clinical diagnosis is based on the history of the individual patient and characteristics of the patient population. Differentiation of scrub typhus from other acute febrile illnesses, such as leptospirosis, murine typhus, malaria, dengue fever and viral hemorrhagic fevers, is difficult due to the similarities of the signs and symptoms (Brown *et al*, 1976, 1984; James, 2000). Currently, laboratory diagnosis of the disease employs immunological techniques such as the Weil-Felix (WF) test, passive hemagglutination assay, indirect immunofluorescence (IF) test, indirect immunoperoxidase (IIP) test and enzyme link immunosorbent assay (ELISA) (Suto, 1980; Yamamoto and Minamishima, 1982; Kelly *et al*, 1988; Kim *et al*, 1993; Suwanabun *et al*, 1997; James, 2000). The WF test has been widely used for clinical identification at hospitals in tropical countries, especially in Thailand. This method is convenient to perform, whereas the IF, IP and ELISA are quite complicated and time consuming (Kelly *et al*, 1988; Kawamura *et al*, 1995;

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Pradutkanchana *et al*, 1997; Kovacova and Kazar, 2000). These immunological methods are limited in their early detection of the illness, since the serum antibodies of the patient are too low.

Nested PCR, a two-step PCR using 2 pairs of primers, has been used for the diagnosis of several infectious diseases (Kelly *et al*, 1990; Furuya *et al*, 1993; Fredricks and Relman, 1999). Molecular diagnostic PCR has been used for the detection of *O. tsutsugamushi* DNA in the blood. In this study, the early diagnosis of scrub typhus in Thailand by the nested PCR method was evaluated by comparing to other routinely used methods (IF and WF test).

MATERIALS AND METHODS

Bacterial strains and media

Three prototype strains of *Orientia tsutsugamusi* (Gilliam, Karp and Kato serotype) and *Rickettsia typhi* Wilmington (courtesy of NIH, Thailand) were propagated in an L 929 cells as previously described (Tamura *et al*, 1984). Fully grown L 929 cells in Eagle's MEM medium (Nissui Pharmaceutical Co.Ltd. Japan) supplemented with 2% fetal calf serum (Gibco BRL, USA) were infected with a suspension of *O. tsutsugamushi*. After a 1-hour incubation at 37ºC in a 5% carbon dioxide incubator, the monolayer was washed with minimal essential medium containing 2% fetal calf serum. The infected monolayer cells were harvested when more than 80% were infected. The purified bacterial DNA was extracted by a QIAgen kit (Qiagen, Hilden, Germany).

Clinical samples

Clinical blood samples were collected from 80 Thai patients (ages 7 to 60 years) who were admitted to hospitals in northern Thailand from January 2000 to September 2001. The patients with suspected scrub typhus infection were diagnosed by clinical symptoms (fever, rash with or without eschar and lymphadenopathy). Control blood samples were collected from healthy blood donors. Acute and convalescent sera (7-10 days after the collection of acute serum) were collected for serological IF and WF tests. The acute clotted blood samples were also collected for PCR testing. The template DNA was extracted from the clotted blood by a QIAgen kit (QIAgen, Hilden, Germany). This study was conducted in accordance with the protocols for human use approved by Medical Research for the Protection of Human Subjects.

Weil-Felix test (WF)

Sera were evaluated to detect the presence of antibodies against the bacterial antigen Proteus OX–K strain, which is common to the antigen of *O. tsutsugamushi*. The agglutination was performed by the stained bacterial antigen suspension kit (Shield the Diagnosis, Dundee, UK). The accepted minimum titer was 40. The sample was considered to be a positive seroconversion, when the titer of the convalescent stage was 160 or more.

Immunofluorescence assay (IF)

Sera were tested for the presence of IgG and IgM against the mixture of *O. tsutsugamushi* prototypes (Gilliam, Karp and Kato serotype) as previously described (Kawamura *et al*, 1995). After the initial dilution of 1:50, the serial 2-fold dilution of the sera was prepared in 0.01 M phosphate saline buffer, pH 7.3. The three pool antigen of Gilliam, Karp and Kato of *O. tsutsugamushi* in a L 929 cell preparation were spotted on a glass slide. The accepted minimum titer was 50. The sample was assigned to be a positive seroconversion, when the titer of IgM or IgG was 400 or more.

Nested polymerase chain reaction (Nested PCR)

PCR was performed as previously described (Furuya *et al*, 1993). Two pairs of primers generating 487 bp were synthesized by the Biosera Unit, Thailand. The primer design was based on the homology of the nucleotide sequence of the gene encoding the 56 kDa major outer membrane protein of *O. tsutsugamushi* (Genbank accession number M33004) (Stover *et al*, 1990). The amplification of the target DNA was done by the nested PCR. The first amplification was carried out using the bracket Primer a (5′-ATTGCTAG TGCAATGTCTGC-3′) region 574-593 and bracket Primer b (5′-CTGCTGCTGTGCTTG CTGCG-3′) region 1568-1587. The second amplification was carried out using a nested Primer c (5′-CCTCAGCCTACTATAATGCC-3′) region

961-980 and nested Primer d (5′-CGACAGATG CACTATTAGGC-3′) region 1428-1447. The PCR amplification mixture (total volume of 50 µl) contained 1.5 mM of $MgCl_2$; 50 mM of KCl; 10 mM of Tris-HCl (pH 8.3) 0.001% (W/V) gelatin; 2.0 µM each of dATP, dGTP, dCTP and dTTP; 0.2 µM primers a and b in the first PCR and 0.2 µM of primers c and d in the second PCR, 1.25 U of the Tag Polymerase (Perkin-Elmer, Cetus, Norwalk) and 5 µl of the template DNA. The mixture was denatured at 94ºC for 30 seconds and annealed at 57ºC for 2 minutes. The chain was extended at 70ºC for 2 minutes in a thermal cycler for 30 cycles. The amplification product was electrophoresed on 1.5% agarose gels, stained with ethidium bromide and observed under ultraviolet transillumination. The sample was considered to be positive when the 487 bp-specific band was detected.

Restriction fragment length polymorphism (RFLP) analysis of the amplification products of *O. tsutsugamushi*

The PCR products were digested with the restriction endonuclease Hae III (Promega Corporation, Madison, USA). Ten µl of amplified PCR product was digested with 10 U of the restriction endonuclease Hae III in 15 µl of reaction volume and incubated at 37ºC for 6 hours. The digested and undigested products were analyzed by electrophoresis in 1.5% agarose gels, stained with ethidium bromide and observed under ultraviolet transillumination.

RESULTS

Weil-Felix test and immunofluorescence assay

The WF test showed 13 positive seroconversions (Proteus OX-K titer ≥ 160) whereas the IF test gave 21 positive seroconversions out of 80 patients (Table 1). High antibody titers (IgM ≥ 400 and/or IgG ≥ 400) were detected in 12 patients. The patients who showed serum antibody titers but were considered as a negative seroconversion are shown in Table 2. Seventeen and 8 seronegative conversion patients were observed by the IF and WF tests, respectively. Forty-two and 54 out of 80 patients showed no antibodies against *O. tsutsugamushi* by the IF and WF tests.

Detection of *O. tsutsugamushi* infection in the early stage of infection by nested PCR, IF and WF

At the early stage of infection, the nested PCR amplified *O. tsutsugamushi* DNA gave 19 positive diagnoses whereas the IF and WF tests showed a positive seroconversion in 13 and 3 out of 80 patients, respectively (Table 3). The amplification product of the target DNA in 1.5% agarose gel electrophoresis showed the 487 bp DNA fragment from the patients in lanes 2, 3 and 4 but no DNA amplification was observed in the healthy blood donor in lane 6 (Fig 1).

Analysis of the PCR product by RFLP

In RFLP, the nested PCR amplified products from the DNA of *O.tsutsugamushi* of about 487 bp were digested by Hae III. Two bands of about 174 and 313 bp were generated. The RFLP profiles of positive strains were identical to the prototype *O.tsutsugamushi*, suggesting that the strains infecting these patients were *O. tsutsugamushi* (Fig 2).

DISCUSSION

The blood samples used in this study were from Thai patients in the northern part of Thailand. Most of them live by agriculture and are subjected to a high risk for scrub typhus infection. A high prevalence of scrub typhus in Thailand has never been reported (Division Of Epidemiology, Thailand 2000) because of the variability of its manifestations and the lack of sensitivity of a laboratory test to confirm the disease. The gold standard for the confirmation of scrub typhus infection is to isolate the organism from the patient (Tamura *et al*, 1984; Kawamura *et al*, 1995; James, 2000) or by other serological methods which are laborious, time consuming and require paired serum for the detection of seroconversion. The conventional WF test, which is the most common method used in Thailand, showed a positive seroconversion for only 13 out of 80 patients (16.25%) (Table 1). The low sensitivity of WF test is due to the low antibody titer against *O. tsutsugamushi*, which was not detectable at the initial stage of hospitalization. The WF test is not a specific serological reaction but a reaction between a *O. tsutsugamushi* specific anti-

Table1
Serum antibody titers for positive seroconversion against of *O. tsutsugamushi* from 80 patients by IF and WF tests.

Patient no.	Days of onset	IF titer[a]				WF titer[a]		
		Ig Class[b]	Early stage[c]	Convalescent stage[d]	Interpretation	Early stage[d]	Convalescent stage[d]	Interpretation
200009	3	M	50	50	Positive	-	-	Negative
		G	100	400				
200013	4	M	50	50	Positive	-	-	Negative
		G	50	400				
200015	5	M	50	100	Positive	-	40	Negative
		G	100	400				
200019	3	M	100	200	Positive	-	-	Negative
		G	200	800				
200023	4	M	50	50	Positive	-	40	Negative
		G	200	800				
200031	4	M	100	50	Positive	40	-	Negative
		G	100	400				
200038	5	M	100	50	Positive	40	160	Positive
		G	200	400				
200042	5	M	100	200	Positive	40	40	Negative
		G	200	400				
200045	6	M	200	100	Positive	40	-	Negative
		G	400	200				
200050	6	M	400	400	Positive	40	160	Positive
		G	400	800				
200053	5	M	400	200	Positive	40	160	Positive
		G	400	800				
200057	3	M	400	800	Positive	40	160	Positive
		G	400	1,600				
200059	5	M	400	800	Positive	80	320	Positive
		G	800	1,600				
200164	5	M	800	400	Positive	40	160	Positive
		G	800	800				
200168	4	M	800	400	Positive	40	160	Positive
		G	400	400				
200172	7	M	400	200	Positive	160	320	Positive
		G	800	1,600				
200004	8	M	400	400	Positive	160	320	Positive
		G	1,600	400				
200174	4	M	400	400	Positive	40	160	Positive
		G	800	800				
200176	6	M	400	800	Positive	40	160	Positive
		G	800	1,600				
200177	9	M	800	400	Positive	160	640	Positive
		G	1,600	1,600				
200180	7	M	400	400	Positive	80	320	Positive
		G	800	1,600				
No. of positive diagnosis					21			13

[a]Reciprocal of the maximum serum dilution; [b]The letters indicates the immunoglobulin class; M: Immunoglobulin M, G: Immunoglobulin G; [c]Acute serum of patients at early stage obtained at the date of admission; [d]Convalescence serum of convalescence stage obtained 7-10 days after the acute serum obtained.

Table 2
Serum antibody titers for negative seroconversion against *O. tsutsugamushi* from 80 patients by IF and WF test.

Patient no.	Days of onset	IF titer[a]				WF titer[a]		
		Ig class[b]	Early stage[c]	Convalescent stage[d]	Interpretation	Early stage[c]	Convalescent stage[d]	Interpretation
200008	3	M	-	50	Negative	-	-	Negative
		G	-	50				
200012	4	M	-	50	Negative	-	40	Negative
		G	50	50				
200028	5	M	-	50	Negative	-	40	Negative
		G	-	-				
200032	1	M	-	50	Negative	-	-	Negative
		G	-	-				
200034	5	M	-	50	Negative	-	40	Negative
		G	-	-				
200036	6	M	-	-	Negative	-	-	Negative
		G	50	100				
200040	5	M	-	50	Negative	-	40	Negative
		G	-	50				
200044	2	M	-	-	Negative	-	-	Negative
		G	-	50				
200048	3	M	-	50	Negative	-	40	Negative
		G	50	50				
200051	1	M	-	50	Negative	-	-	Negative
		G	-	50				
200054	3	M	50	100	Negative	-	40	Negative
		G	50	100				
200056	4	M	50	100	Negative	-	40	Negative
		G	-	50				
200162	2	M	-	50	Negative	-	-	Negative
		G	50	50				
200166	3	M	-	50	Negative	-	-	Negative
		G	-	50				
200169	6	M	-	-	Negative	-	80	Negative
		G	50	50				
200173	2	M	-	-	Negative	-	-	Negative
		G	-	50				
200179	2	M	-	50	Negative	-	-	Negative
		G	-	-				
No. of negative diagnosis					17			8

[a]Reciprocal of the maximum serum dilution; [b]The letter indicates the immunoglobulin class; M: Immunoglobulin M, G: Immunoglobulin G; [c]Acute serum at early stage obtained at the date of admission; [d]Convalescence serum at convalescence stage obtained 7-10 days after the acute serum obtained.

body and a component of Proteus OX-K which is common to *O. tsutsugamushi* (Kawamura *et al*, 1995; Pradutkanchana *et al*,1997; Kovacova and Kazar, 2000). The WF test exhibited a sensitivity of only 61.90% (13/21) when compared to IF, the reference standard for serogical diagnosis. IF has poor sensitivity in the early stage of illness because the antibody is too low to be detected (Table 3). The IF test is hampered by the multiple serotypes of *O. tsutsugamushi*, resulting in antigen

Table 3
Comparison of the early diagnosis of scrub typhus from 23 out of 80 patients by IF, WF and nested PCR in the early stage infection.

Patient No.	Day of illness	Early stage		
		IF	WF	PCR
200054	3	-	-	+
200056	4	-	-	+
200009	3	-	-	+
200013	4	-	-	+
200015	5	-	-	+
200019	3	-	-	+
200023	4	-	-	+
200031	4	-	-	+
200038	5	-	-	+
200042	5	-	-	+
200045	6	+	-	+
200050	6	+	-	+
200053	5	+	-	+
200057	3	+	-	+
200059	5	+	-	+
200164	5	+	-	+
200168	4	+	-	+
200172	7	+	+	-
200004	8	+	+	+
200174	4	+	-	+
200176	6	+	-	-
200177	9	+	+	-
200180	7	+	-	-
No. of positive diagnosis		13	3	19

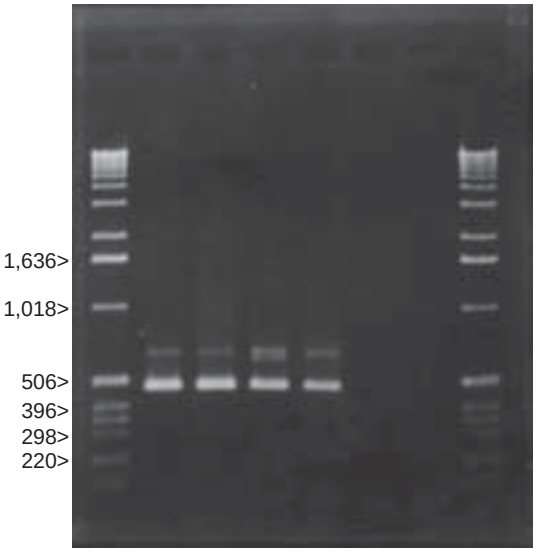

Fig 1–Detection of the 487 bp DNA encoding for 56 kDa of *O. tsutsugamushi* by nested PCR using 1.5% agarose gel electrophoresis. DNA extracted from the suspected scrub typhus patients No. 200054, 200056 and 200004 (lanes 2, 3 and 4), health blood donor (lane 6), Control *O. tsutsugamushi* serotype Karp (lane 5), Control *R. typhi* (lane 7). Lanes 1 and 8 contained a 1-kb DNA ladder as a size marker (GIBCO BRL, Inc) The numbers on the left are the sizes of base pairs.

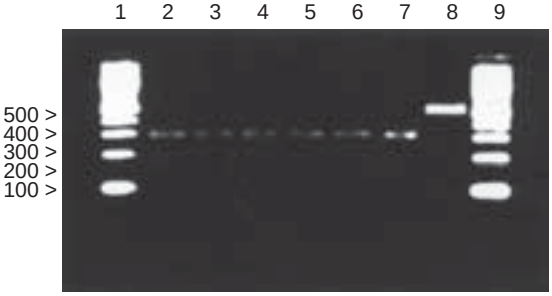

Fig 2–Restriction endonuclease profile of DNA amplified from patients and prototype of *O. tsutsugamushi* digested with Hae III. *O. tsutsugamushi* (lane 2) and sample from patients (lanes 3,4,5,6 and 7) revealed two bands of about 174 and 313 bp. Undigested PCR product of *O. tsutsugamushi* were about 487 bp (lane 8). Lanes 1 and 9 contained a 1 kb DNA ladder as size marker (BioLabs, Inc). The numbers on the left are sizes of base pairs.

diversity between the species (Shirai *et al*, 1979; Suwanabun *et al*, 1997; James, 2000). In the seroprevalence study, 38 out of 80 patients (47.50%) were positive. This indicates scrub typhus in endemic in northern Thailand. The diagnosis of scrub typhus via serological methods is time consuming and it is detected only after the acute illness has resolved. Fatal, multiorgan complications or permanent disability in some patients can be a result of delayed treatment (Silpapojakul, 1997). An early, precise diagnosis is needed for the proper management of scrub typhus.

In this study, *O. tsutsugamushi* DNA could be detected as early as day 3 of fever even before the appearance of a specific antibody in the blood by the nested PCR technique (Table 3). Patients in Thailand sometimes seek medical care rela-

tively late in their illness. Those who visit the hospital after 7 days of fever and have high antibodies can be diagnosed by the IF and WF tests. The amplification of Oriential DNA from 10 and 20 patients with negative serocoversion towards *O. tsutsugamushi* by IF and WF suggested an efficient detection of early infection. With the IF test, 4 patients (No. 200172, 200176, 200177 and 200180) showed high antibody titers, while the nested PCR showed a negative result. This may be due to the clearance of the immune system (Harson, 1992). The PCR is therefore useful for early detection of the infection since the sensitivity and specificity of the nested PCR in negative seroconversion was high enough to use for diagnosis. For the reference standard serological method using paired sera, the IF test showed positive seroconversion in 21 out of 80 patients. In the early stage, the IF test showed a positve result for only 13 patients whereas the PCR could detect *O. tsutsugamushi* DNA in 19 patients. The sensitivities of PCR, IF and WF at the early stage of infection compared to the reference standard method, IF test were 90.47% (19/21), 61.90% (13/21) and 14.28% (3/21) respectively. The paired sera of patients No. 200054 and 200056 were not diagnosed by IF or WF but were diagnosed by nested PCR. Detection by the nested PCR technique is rapid, not difficult for practical use and can be performed in 1 day on admission whereas IF and WF take 10 -14 days to investigate the convalescence serum before obtaining a precise diagnosis. In additon, RFLP was used to identify Oriential amplified DNA. The RFLP profiles of these patients and *O. tsutsugamushi* were identical, suggesting that the strains infecting these patients were *O. tsutsugamushi*. The Oriential DNA was detectable in 19 of 23 patients by nested PCR. The excellent sensitivity and specificity of the nested PCR compared to IF and WF tests suggests that nested PCR could be a specific, rapid assay for the early detection of *O. tsutsugamushi* infection. However, the serological test is still an appropriate technique when antibodies are high enough. Therefore, integration of the nested PCR technique resulted in the development of a diagnostic system for scrub typhus, especially in the endemic regions. In conclusion, an early diagnosis for the routine detection of *O. tsutsugamushi* from suspected patients during the acute phase should be done by the nested PCR in concomitantly with the serological method.

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