5. RESEARCH DESIGN AND METHODS

1. Research design

The blood samples were collected from the patients who were clinically suspected of scrub typhus from hospitals of 8 Regional Medical Sciences Centers in Thailand. . The sera were tested for the presence of IgG and IgM against the *O. tsutsugamushi.* The positive *O. tsutsugamushi* seroconversion samples were investigated for the genotypes and nucleotide sequence polymorphism of the 56 kDa protein gene. The DNA was extracted by QIAgen kit (Qiagen, Hilden Germany) and used as a template for PCR. The 56 kDa protein gene was the target for the amplification. For genotyping, the PCR products were analyzed by nested PCR using serotype specific primer. The amplified products were visualized on 1.5 % agarose gel electrophoresis and compared with the amplified product of reference serotypes. To ensure that the genotyping obtained from nested PCR and the characterization of nucleotide sequence polymorphism of the 56 kDa protien gene were correct , the PCR products were purified and subject to the cycle sequencing using the ABI PRISM™ dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Applied Biosystems, USA). The sequencing product were purified prior to being injected into the ABI 310 automated DNA sequencer (Perkin-Elmer, Applied Biosystems, USA) and analyzed by the computer programmes; ABI 310 data collection version 3.10 and ABI 310 DNA sequencing version 2.2. Finally, the 56 kDa protein gene sequencing data from each sample was compared with the nucleotide sequence of the reference strain, obtained from the GenBank database by using NCBI-BLAST network service of the National Center for Biotechnology Information, Maryland , USA.

2. MATERIAL AND METHODS

2.1 Clinical sample

Blood samples were collected from 300 Thai febrile patients (age from 7 years to 60 years) admitted to the hospitals at 8 Regional Medical Sciences Centers of Thailand from January 1999-December 2002. The patients were diagnosed as scrub typhus based on Immunofluorescence (IF) test. Clotted blood sample were collected for PCR testing.This study was conducted in accordance with protocol approved for medical research on human subjects.

2.2 Bacterial strain and media

Rickettsia : Three prototype strains of *Orientia tsutsugamushi* (Gilliam, Karp and Kato serotype)and *Rickettsia typhi* Wilmington were courtesy of National Institute of Health, Thailand. They were propagated in L 929 cell as described by Tamura et al., (62).

The L 929 cell line was infected with *O. tsutsugamushi* suspension. After 1 hour at 37 °C , the monolayer was washed with minimal essential medium (Nissui Pharmaceutical Co. Ltd., Japan) containing 2 % fetal calf serum (Gibco BRL, USA) and incubated in an atmosphere of 5 % CO_2 and 95% air. The infected monolayer was harvested when the cytopathic effects were more than 80%. The DNA of *O. tsutsugamushi* was extracted by using QIAgen kit (Qiagen, Hilden Germany).

2.3 Laboratory diagnosis

2.3.1 Immunofluorescence assay The 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 (5). After the initial dilution of 1:50, the serial 2-fold dilution of the sera was prepared in 0.01 M phosphate buffer saline (PBS), pH 7.3. The three pool antigens of Gilliam. Karp and Kato of *O. tsutsugamushi* in L 929 cell preparation were spotted on the glass slide. The accepted minimum titer was 50. The sample was assigned to be positive seroconversion. when the titer of IgM or IgG was 400 or more (83). For serodiagnostic methods, a second serum sample should be taken after 7-10 days, the increase rising antibody titer between paired sera should be examined to ensure a correct positive diagnosis. However, in early stage of infection. the single specimen was assigned positive when the titer of IgM or IgG was 400 or more.

Procedure for IFA:

1. *O. tsutsugamushi* antigens was prepared and smeared on the glass slide, dried and treated with cold acetone for 15 minutes.
2. About 10 µl of diluted test sera was overlaid onto spot of *O. tsutsugamushi* smears.
3. The slide is incubated in 37 °C for 30 minutes in moist chamber.
4. The slide is washed by immersion in PBS in a slide staining dish and placed on a vibrating plate, with gentle movement. The PBS was changed three times at 5 minutes intervals

5. The slide was dried in air stream.

6.The spots on the slide were overlaid with 10 µl of appropriately diluted biotinylated anti- immunoglobulin antibody.

7. Repeated step 3, 4 and 5.

8.The spots on the slide are overlaid with 10 µl of appropriately diluted FITC conjugated avidin at 37 °C for 10 minutes.

9. Repeated step 3,4 and 5.

10.The spots on the slide are mounted with glycerol buffer.

11.The slide was observed under fluorescence microscope.

2.3.2 Weil-Felix test The sera were tested for the presence of IgG against the Proteus OX-K that is common to *O. tsutsugamushi.* After the initial dilution of 1:40, the serial 2-fold dilution of the sera was prepared in 0.01 M phosphate buffer saline (PBS), pH 7.3. The accepted minimum titer was 40. The sample was assigned to be positive seroconversion when the titer of IgM or IgG was 160 or more (83). For serodiagnostic methods, a second serum sample should be taken after 7-10 days, the increase rising antibody titer between paired sera should be examined to ensure a correct positive diagnosis. However, in early stage of infection, the single specimen was assingned positive when the titer of IgM or IgG was 160 or more.

2.3.3 Nested PCR

2.3.3.1DNA extraction The DNA of *O. tsutsugamushi* and the template DNA were extracted from clotted blood by QIAgen kit (Qiagen, Hilden Germany).

2.3.3.2 Primer

(1). Oriential specific primers The two pairs of oligonucleotide primers were designed based on the nucleotide sequence of gene encoding the 56 kDa protein of *O. tsutsugamushi* which could amplify all *O. tsutsugamushi* serotype. The 56 kDa protein gene was amplified using outer universal primers: primer a and primer b in the first PCR and then using inner or nested universal primer: primer c and primer d in the second PCR. Sizes of PCR products, when amplified with the outer and inner universal primers, were expected to be 487 basepair (bp). The nucleotide sequence of primers and the location of those in the 56 kDa protein gene are shown in Figure 2.

Primer a 5′-ATTGCTAGTGCAATGTCTGC-3′

Primer b 5′-CTGCTGCTGTGCTTGCTGCG-3′

Primer c 5′-CCTCAGCCTACTATAATGCC-3′

Primer d 5′-CGACAGATGCACTATTAGGC-3′

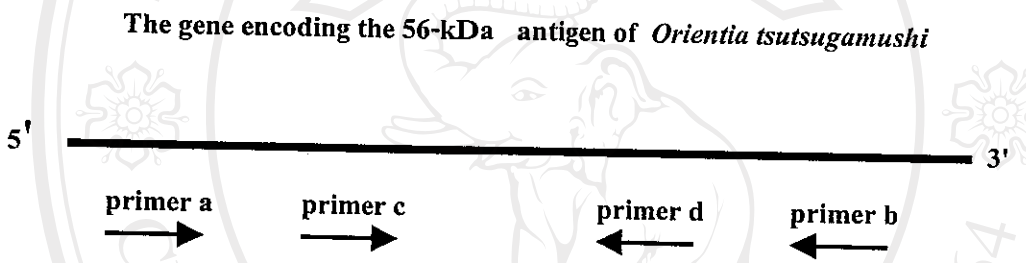

Figure 2 The oligonucleotide sequences of the universal primers; primer a,b and primer c and d and their position on 56 kDa protein gene of *O. tsutsugamushi*.

(2). Serotype specific primers The oligonucleotide serotype specific primers were designed based on the nucleotide sequence of gene encoding the 56 kDa protein of *O. tsutsugamushi* which could amplify *O. tsutsugamushi* Gilliam, Karp and Kato serotype. The 56 kDa protein gene was amplified using universal primer a and primer b in the first PCR and primer c and serotype specific primer in the second PCR, which generated the 410, 220 and 232 bp size specific bands of Gilliam, Karp and Kato serotype, repectively. The nucleotide sequence of serotype specific primers and the location of those in the 56 kDa protein gene are shown in Figure 3.

primer G 5′-CTTTATACACTATATATCTT-3′ specific to the Gilliam serotype

primer KP 5′-ACAATATCGGATTTATAACC-3′ specific to the Karp serotype

primer KT 5′-GAATATTTAATAGCACTGGC-3′ specific to the Kato serotype

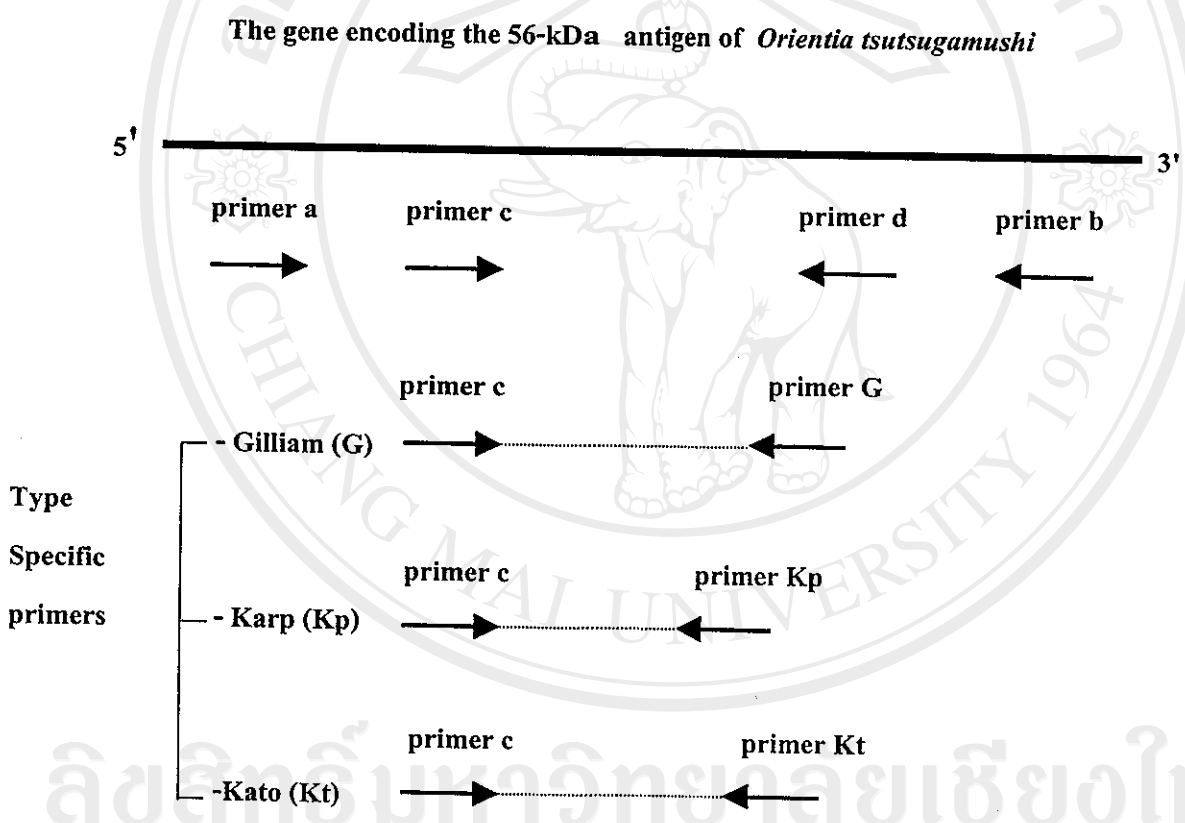

Figure 3 The oligonucleotide sequences of the serotype specific primers and their position on 56 kDa protein gene of *O. tsutsugamushi.*

2.3.4 Polymerase chain reaction (PCR)

Nested PCR was performed as described by Furuya (79).Two pairs of primers based on the nucleotide sequence of gene encoding the 56 kDa protein of *O. tsutsugamushi* (GenBank accession number: M33004)as described by Stover were designed (61). The first amplification was carried out using primer a (5′-ATTGCTAGTGCAATGTCTGC-3′) region 574-593 and primer b (5′-CTGCTGCTGTGCTTGCTGCG-3′) region 1568-1587. The second amplification was carried out using primer c (5′-CCTCAGCCTACTATAATGCC-3′) region 961-980 and primer d (5′-CGACAGATGCACTATTAGGC-3′) region 1428-1447. The sample was identified as positive when the 487 bp-specific band was detected. For Gilliam, Karp and Kato serotype identification, the second amplification was carried out using primer c (5′-CCTCAGCCTACTATAATGCC-3′) region 961-980 and primer specific to each serotype. Primer G (5′-CTTTATATCACTATATATCTT-3′), specific to the Gilliam serotype ; primer Kp(5′-ACAATATCGGATTTATAACC-3′), specific to the Karp serotype and primer Kt,(5′-GAATATTTAATAGCACTGGC -3′), specific to serotype specific to Kato serotype correspond to regions 1350 —1371, 1160-1181 and 1172-1193 respectively. The samples were identified as positive for Gilliam, Karp and Kato serotype when the 410, 220 and 232 bp size specific bands were detected, respectively. 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 ; 2.0 µM each of dATP, dGTP, dCTP and dTTP; 0.2 µM primers a and b in first PCR and 0.2 µM primers c and d in second PCR , 1.25 U of Tag Polymerase (Perkin-Elmer Cetus, Norwalk ,USA) and 5 µl of template DNA. For serotype identification 0.2 µM primer c and specific serotype primer were employed in second PCR. The mixture was denatured at 94 °C for 30 seconds, annealed at 57 °C for 2 minutes and the chain was extended at 70°C for 2 minutes in a thermal cycler for 30 cycles.

2.3.5 Detection of the PCR product

Ten µl of the amplified product was electrophoresed on 1.5 % agarose gels in TBE buffer at 100 volts for 45 minutes. The gel was stained with ethidium bromide for 15 minutes, destained with distilled water for 15 mintures and visualized under ultraviolet transillumination. The DNA bands were compared with a 1 Kb DNA Ladder marker (GIBCO-BRL, USA).

2.3.6 Dye terminator cycle sequencing technique

The nucleotide sequence of the 56 kDa protein gene of the *O. tsutsugamushi* was determined by using the ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Applied Biosystem, USA). This ready reaction kit consisted of the unique properties of AmpliTag DNA polymerase for dye terminator sequencing with the convenience of ready reaction format. In this kit, the same ready to use reagents was included in a single tube. The reagents were suitable for performing fluorescence-based cycle sequencing reactions of PCR products. The nucleotide sequence target was amplified using the PCR technique with *Tag* DNA polymerase. The PCR product was purified by the Glass MAX spin Catridge System (Gibco-BRL, USA). Approximately, 100-180 ng of purified template was mixed with 8.0 µl of terminator premix containing A-dye T, C-dye T, T-dye T, G-dye T, dNTPs, Tris-HCl (pH 9,0), $MgCl_2$, thermal stable pyrophosphatase and AmpliTag DNA polymerase. The mixture was mixed and placed in programmable heat block of the GeneAmp 2400 thermocycler (Perkin-Elmer, Cetus, USA). The reaction was run for 25 cycles. Each cycle was performed in 3 steps, denaturation at 96 °C for 10 seconds, primer annealing at 50 °C for 5 seconds and extension at 60 °C for 4 minutes.

2.3.7 Purification of sequencing products

The sequencing products was purified from the reaction mixture by using the ethanol precipitation. Two µl of 3M sodium acetate pH 4.6 and 50 µl of 95% ethanol were added and placed on ice for 10 minutes, centrifuged at 10,000 rpm at 4 °C for 30 minutes. The supernatant was then removed and air dried for 30 minutes at room temperature. The pellet was kept at -20 °C until use.

2.3.8 ABI 310 automated sequencer

The pellet of purified sequencing product was resuspended in 25 µl of Template Suppression Reagent: TSR (Perkin-Elmer, Applied Biosystems, USA). The tube was mixed and centrifuged before heating at 95 °C for 2 minutes and placed on ice. It was then put into the ABI 310 automated DNA sequencer (Perkin-Elmer, Applied Biosystems, USA).

The fluorescence emitted from the dye-labeled DNA fragments was detected by passing it through the capillary electrophoresis using POP6 sequencing gel (Perkin-Elmer, Applied Biosystems, USA). The capillary electrophoresis was performed at 12.2 KVs, at 50°C for 120

minutes. The withdrawn fluorescence was collected and transferred into digital signals, then analyzed automatically by the computer programmes ABI 310 data collection version 3.10 and ABI 310 DNA sequencing version 2.2. The data showed the nucleotide sequences with the electrophoregram. Analysis of the derived nucleotide sequences was performed by NCBI-BLAST network service of the National Center for Biotechnology Information, Bethesda , Maryland, USA.