Genotype Diversity and Distribution of *Orientia tsutsugamushi*

causing Scrub Typhus in Thailand

Toon Ruang-areerate¹, Pimmada Jeamwattanalert², Wuttikorn Rodkvamtook¹, Allen L. Richards³, Piyanate Sunyakumthorn⁴ and Jariyanart Gaywee¹

Research Division¹, and Department of Enterics Disease², Armed Forces Research Institute of Medical Science, Bangkok 10400, Thailand; Viral and Rickettsial Diseases Department, U.S. Naval Medical Research Center, Silver Spring, MD 20910, USA³; Department of Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803, USA⁴

**RUNNING TITLE:** Thai *Orientia tsutsugamushi* genotype and distribution

**Address for correspondence:** Jariyanart Gaywee, Epidemiology Section, Research Division, Armed Forces Research Institute of Medical Sciences (AFRIMS) 315/6 Rajvithi Road, Bangkok 10400, Thailand. Phone: 66 2 644 4888 ext. 2151. Fax: 66 2 644 6765. E-mail: jarivanartg@afrims.org

Toon Ruang-areerate, Epidemiology Section, Research Division, Armed Forces Research Institute of Medical Sciences (AFRIMS) 315/6 Rajvithi Road, Bangkok 10400, Thailand. Phone: 66 2 644 4888 ext. 2153. Fax: 66 2 644 6765. E-mail: toonr@afrims.go.th, youangtr@yahoo.com
Scrub typhus caused by antigenically disparate isolates of *Orientia tsutsugamushi* is a widely distributed mite-borne human disease in the Asia Pacific region. Information regarding heterogeneity of the immunodominant 56 kDa type specific antigen (TSA) gene is crucial for the design and evaluation of scrub typhus-specific diagnostic assays and vaccines. Using IFA and PCR assays, *O. tsutsugamushi* was detected in fever of unknown origin patients and rodents obtained from 6 provinces of Thailand during 2004–2007. Sequences were determined for a fragment of the 56 kDa TSA gene and the relationship between these sequences and those previously determined were assessed. The phylogenetic analyses of partial 56 kDa TSA gene sequences demonstrated wide diversity and distribution of *O. tsutsugamushi* genotypes in Thailand. Furthermore, the genetic diversity grouped the scrub typhus agents into 2 commonly and 5 infrequently found genotypes within 6 provinces of Thailand. The two most commonly found genotypes of *O. tsutsugamushi* described in this study do not associate with the prototype strains that are widely used for design and evaluation of diagnostic assays and vaccine candidates. Thus, these new genotypes should be considered for future scrub typhus assay and vaccine development.

**KEYWORDS:** *Orientia tsutsugamushi*; scrub typhus; diversity; distribution; transmission; vaccine; diagnostic assay; Thailand
**Orientia tsutsugamushi**, formerly known as *Rickettsia tsutsugamushi*, is the causative agent of scrub typhus, a major cause of acute febrile illnesses in rural southeast Asia (20, 30). This obligate intracellular Gram-negative rod shaped bacterium (16) is vertically maintained in *Leptotrombidium* spp. mite populations and transmitted to humans by the bite of infected larval stage mites called chiggers (30). The pathogen may also be transmitted horizontally between mites and infected vertebrate hosts (10, 11).

This mite-borne disease is found widely distributed across the Asia Pacific region and causes substantial morbidity in an area stretching from Pakistan, Australia, Japan, Korea to Thailand (14). This endemic region is often referred to as the tsutsugamushi triangle which hosts approximately 1 billion people (5). Currently, there is no vaccine available against scrub typhus, thus, effective management of this disease relies upon rapid diagnosis and antibiotic therapy with either doxycycline, tetracycline or chloramphenicol (30).

Antigenic variation among isolates of *O. tsutsugamushi*, due to the immuno-dominant 56 kDa type specific antigen (TSA) located on the surface of *O. tsutsugamushi* (29), is commonly and widely used for *O. tsutsugamushi* serotyping and strain classification (2, 3, 13, 14, 26, 28). Originally, there were three distinctive antigenic prototypes of *O. tsutsugamishi* (Karp, Kato and Gilliam) described (7). New isolates of *O. tsutsugamishi* were therefore classified on the basis of reactivity with hyperimmune serum raised against these prototype strains (3). Later, additional antigenic variation of different serotypes were found in many countries (20). Thus, serotyping of new isolates are currently carried out by immuno-fluorescence assays using strain- or type-specific monoclonal antibodies or hyperimmune sera which recognize the 56 kDa TSA of several unique strains of *O. tsutsugamushi*.

Serotyping is limited by the need for well characterized strains to produce the required reagents and the breadth of reactivity to detect new strains (9). These antigenic variations were linked to sequence diversity of 56 kDa TSA gene (18).
Recently genotyping of *O. tsutsugamushi* has been used to characterize unique isolates. This has been accomplished by restriction fragment length polymorphism (RFLP) or sequence analysis of 56 kDa TSA gene or gene fragment polymerase chain reaction (PCR) amplicons (17, 29). In contrast to serotyping, molecular methods (8, 18) can be used to determine evolutionary relationships among the different genotypes. This useful information gained regarding genetic diversity and phylogenetic relationships of *O. tsutsugamushi* based on 56 kDa genotyping is crucial for determining the breadth of antigenic heterogeneity which is critical for the development of sensitive and specific diagnostic assays as well as effective vaccine candidates.

Utilizing sequences recently obtained from 56 kDa TSA gene of human and rodent derived *O. tsutsugamushi*, we discovered genetic diversity that grouped the scrub typhus agent into 2 commonly and 5 infrequently found genotypes located in 6 provinces of Thailand. The detection of identical genotypes in different hosts including humans implies that potential transmission of scrub typhus may occur in any area where there is an appearance of one of infected hosts. The commonly identified genotypes described herein do not phylogenetically associate with the prototypes (Karp, Kato and Gilliam), therefore, the design and evaluation of scrub typhus diagnostic assays as well as vaccines candidates which relies on original prototypes may not be effective in Thailand.
MATERIALS AND METHODS

Sample collection. A total 604 fever of unknown origin (FUO) patients’ blood samples were obtained from clinical centers in central (Bangkok; 203 cases), northeastern (Nakhonratchasima, Sisaket, Sakonnakhon; 183 cases) and northern (Chiangmai, Tak; 218 cases) regions of Thailand between February 2004 and April 2007. Patients provided informed consent before their blood samples were collected and used in this study. Blood samples were subsequently shipped at ≤−70°C to the Armed Forces Research Institute of Medical Sciences, Bangkok (AFRIMS) for further studies. This study was approved by the Institutional Review Board, Royal Thai Army Medical Department, Thailand. Culture isolates of O. tsutsugamushi were obtained from 3 of 69 rodents collected in Phangna (southern region of Thailand), 2006 using the methods described previously (25). All captured rodents were processed following appropriate laboratory animal procedures (6). This study was approved by the Animal Research Committee of the Royal Thai Army Component, Armed Forces Research Institute of Medical Sciences.

Indirect fluorescence antibody assay (IFA). All human blood samples were initially tested for O. tsutsugamushi infection using IFA (4, 24). Briefly, pooled antigens from the prototype strains (Karp, Kato and Gilliam) cultured and passaged in mouse fibroblast cell line (L929) were used to screen and detect O. tsutsugamushi-specific antibodies. FITC conjugated to polyclonal rabbit anti-human immunoglobulins: IgM and IgG (Dako, Glostrup, Denmark), were used to detect O. tsutsugamushi specific antibodies in humans. Serum samples were initially screened against the pooled antigens at 1:50 dilution. If the serum samples showed positive reactivity with O. tsutsugamushi antigen, the titer of antibodies in those samples was determined using a series of two-fold dilutions of 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400 and 1:12800 of the sera in PBS. Positive and negative controls were used in all tests.
and the cut-off titer of 400 was used to identify seropositivity. Pair serum samples were not available from the FUO patients.

**PCR and DNA sequencing.** Genomic DNA of FUO patients’ EDTA blood and culture isolates from rodents were extracted using DNeasy blood kit and DNeasy tissue kit (QIAGEN, Hilden, Germany), respectively. The nested PCR corresponding to 56 kDa TSA gene spanning 3 of the 4 major variable regions was performed by utilizing two sets of primers: the outer primers (JG-OtF584, 5′-CAA TGT CTG CGT TGT CGT TGC; RTS9, 5′-ACA GAT GCA CTA TTA GGC AA) and inner primers (F, 5′-AGC GCT AGG TTT ATT AGC AT and RTS8, 5′-AGG ATT AGA GTG TGG TCC TT). The PCR profile was:

- Denaturation at 94°C for 3 min, follow by 40 cycles of 94°C for 30 sec, 57°C for 40 sec and 72°C for 1 min, and final incubation at 72°C for 7 min.

PCR products of expected amplicon’s size were purified using High Pure PCR Template Preparation kit (Roche, Indianapolis, IN, USA). The purified PCR products were directly sequenced using dye terminator method (BigDye® Terminator sequencing kit; Applied Biosystems, Foster City, CA, USA) by ABI PRISM™ 377 DNA sequencer (Applied Biosystems).

**Phylogenetic analysis.** Consensus sequences were constructed using sequencher 3.1 (GeneCodes, Ann Arbor, MI, USA). The 56 kDa TSA gene identification was done by BLAST based on pairwise similarity search (http://ncbi.nlm.nih.gov/blastn) (1). Nucleotide identity was calculated to sort and group closely related *O. tsutsugamushi* (>99%) using MegAlign tool of Lasergene, DNASTar® (DNASTAR Inc., Madison, WI, USA). The selected sequences have been deposited in GenBank under accession numbers (Table 1). Also shown in Table 1 are four 56kDa TSA partial gene sequences of culture isolates from rodents collected in our previous study: Chonburi, eastern region of Thailand (25), that were included in the analysis of this study. Nucleotide sequences of human and rodent samples were aligned with reference *O. tsutsugamushi* sequences (3, 8, 23) using MegAlign tool of Lasergene,
DNAstar® by Cluster W algorithm based on amino acid translation. Neighbor-joining (NJ), Maximum parsimony (MP) and Maximum likelihood (ML) methods were used to generate phylogenetic relationships using PAUP 4.0b10 (27). The overall transition-transversion ratio was calculated using the general time-reversible method (31) chosen by MODELTEST 3.06 (21, 22).
RESULTS AND DISCUSSION

Initial IFA demonstrated that antibodies specific for *O. tsutsugamushi* were found in 4.8% (IgM) and 4.3% (IgG) of the FUO patients’ blood samples (*n* = 604), whereas, detection of *O. tsutsugamushi* DNA was found in 7.95% of these samples (Table 2). The highest infection prevalence was found in samples from the northern provinces (*n* = 218; IFA, IgM = 11.98% and IgG = 10.55%; PCR = 20.64%) whereas evidence of infection was rarely found in samples from the central (*n* = 203; IFA, IgM = 0.49% and IgG = 0.49%; PCR = 0.49%) and northeastern provinces (*n* = 183; IFA, IgM = 1.09% and IgG = 1.09%; PCR = 1.09%) (Table 2). These data corresponded with incidence rates reported for scrub typhus during 2004–2007 (Data from Center of Epidemiological Information, Bureau of Epidemiology, by Ministry of Public Health). The concordance of incidence cases reported and this study indicate that scrub typhus is common and mostly found in the northern region of Thailand. According to non-normal distribution of the samples (Table 3) (a, b, and c; Kolmogorov-Smirnor test, *P* < .001, respectively), Mann-Whitney *U* test (*U*) was employed for statistic analysis between different methods of northern samples. The detection efficiency using IFA (IgM\(^a\), 11.93% and IgG\(^b\), 10.55%) was significantly lower than PCR\(^c\) (20.64%) (a, b vs. c; *U*, *P* = .031, .01, respectively) but there was no significant difference between IgM\(^a\) and IgG\(^b\) (a vs. b; *U*, *P* = .666). The comparison demonstrates that PCR can detect *O. tsutsugamushi* infection better than IFA when size of the infected population is increasing, e.g., northern provinces. Thus, the PCR assay is a good alternative choice for scrub typhus detection, especially, when conventional IFA cannot prove current infection.

Genetic characterization of 51 consensus sequences of the partial 56 kDa TSA gene showed that the human (*n* = 48) and rodent (*n* = 3) samples were highly similar to 56 kDa TSA gene of *O. tsutsugamushi* listed in GenBank. The selected sequences (Table 1, *n* = 24) were aligned with 46 reference *O. tsutsugamushi* sequences retrieved from GenBank. Figures
1 (1A and 1B) and 2 illustrate phylogenetic relationships generated by NJ, MP and ML methods. Tree topologies generated by three methods show concordance of node divergence except that there is an exchange between Shimokoshi and Kato clades (Fig. 1A and 1B) in NJ tree but not for MP and ML trees (Fig. 1B and 2). Low bootstrap value (<50%) is responsible for the occurrence of uncertainty divergence of these two nodes. The phylogenetic trees generated by three methods have similar tree topology and demonstrate that, in 6 provinces of Thailand, 56 kDa TSA gene of *O. tsutsugamushi* are diverse and form polyphyletic evolution. The limitation of the use of hypervariable regions based on three of the four major variable regions (VD I, II and III) of 56 kDa TSA gene might potentially represent an even more hypervariation than whole gene itself. Nevertheless, topology based on these 3 regions does not significantly differ from whole gene topologies employing the same method (Fig. 1A) (8, 15). Slight differences in topology show that there may have been an exchange between Gilliam and Kato assemblages which was observed in some works with low bootstrap value (<50%) (3). Thus, this may be responsible for uncertainty divergence. We have chosen ML tree for describing gene evolutionary relationships among *O. tsutsugamushi* because the ML tree is generated based on evolutionary and tree searching methods which best meets optimality criterion by evaluating individual trees (12).

Figure 2 illustrates an enlargement ML tree in which human and rodent-infected *O. tsutsugamushi* in six provinces are labeled with assigned genotypes. The newly characterized genotypes of closely related *O. tsutsugamushi* (including human and rodent strains) obtained from different Thai provinces are listed in table 1 and shown to cluster into 7 assemblages (Fig. 2). Two assemblages are located within the well-known LA and KATO genotypes (8) whereas five assemblages cluster in distinctive genotypes that we have named as SEA1, SEA2, SEA3, TH1 and TH2 according to original location and distribution. Four of these assemblages cluster near the genotypes Karp (SEA1 and SEA2), Ikeda (SEA3) and UT302.
(TH2). The genotype SEA1, SEA2, Karp and LA form monophyletic divergence indicating a common ancestor sharing and close relationships among these *O. tsutsugamushi*. Therefore, SEA1 should be Karp-related. The trees in this study have demonstrated that SEA2 clustered closer to LA which was reported previously as a different genotype (8) than Karp prototype (figure 1 and 2). Thus, it might be more appropriate that “LA-related” should be assigned for SEA2 than “Karp-related”. SEA3 forms monophyletic with Ikeda group which has been previously denoted as JG type (14), so that, JG-related should be applied for SEA3. In contrast to the other genotypes, TH1 formed a separately deep node divergence distantly but linked to Kato and LF-1 genotype.

Interestingly, the previous reported strains, e.g., UT76, UT176, UT418 (3) which were classified as Karp-like are more closely associated with SEA1, SEA2 and LA. These results suggest that these strains should belong to SEA1, SEA2 and LA rather than the Karp genotype. Nucleotide identity of these UT genotypes to Karp was lower than 95% (3) which corresponded to our analysis. Genotype classification of *O. tsutsugamushi* isolates in a Karp-like grouping is unclear and complicated by the numerous disparate isolates recently identified and therefore the definition of “Karp-like” strains continues to evolve (9, 14).

The prevalence of *O. tsutsugamushi* genotypes described herein appear to be either common (SEA2 and SEA3) or infrequent (KATO, LA, SEA1, TH1 and TH2). The most commonly found genotypes, SEA2 and SEA3 (*n* = 23 and *n* = 16, respectively), collectively encompassed 81.25% of *O. tsutsugamushi* causing scrub typhus illness (*n*/N = 39/48) described in this study whereas the other five genotypes collectively were only responsible for 18.75% of the scrub typhus cases. The use of specific reagents to different genotypes will increase the specificity and sensitivity of *O. tsutsugamushi* detection in scrub typhus patients (9). Therefore, development of vaccines and diagnostic kits, especially those to be used in Thailand, should take into account these *O. tsutsugamushi* genotypes.
There appears to be no occurrence of geographic restriction among *O. tsutsugamushi* within 6 provinces of Thailand. The *O. tsutsugamushi* collected from the same location did not necessarily cluster together in the same genotype (Fig. 2). Moreover, different genotypes were variously distributed in 6 provinces (Fig. 3). In addition, to a lack of correlation between location and genotype there was no correlation between host and genotype. For example the *O. tsutsugamushi* isolates from rodents (CB62r-ER, CB52r-ER) (25), humans (UT76, CM464h-NR, MH650h-NR) (3), and a mite (TWYu8-1) (23) were obtained from distinct locations but grouped together in SEA2 and share the same divergence assemblage with high nucleotide and amino acid similarity (100% identity). The similarity of a genotype in different hosts including humans implies that potential scrub typhus transmission may occur in any area where there is an appearance of one of the infected hosts.

Parola et al. (2008) have demonstrated that specific antigens, i.e., Gilliam, Kato and Kawasaki, were cross reactive against antibodies derived against different strains identified in Laos patients’ sera. The different strains were closely related based upon the 56 kDa TSA gene sequence to previous work (3, 23) and clustered within new genotypes. The occurrence of cross-reactive antibodies to different serotypes has been observed in many studies (9, 19), however, this may not always be the case as when infection occurs with diverse strains that are not included in the antigen panel (9). Therefore, specific antigens including the three prototypes, i.e., Karp, Kato and Gilliam, and new genotypes that were observed in this study should be considered for testing against any Thai patients’ sera to ensure detection of antibodies produced to different *O. tsutsugamushi* serotypes in the future.

In this study, we have demonstrated 56 kDa TSA genotype diversity in 6 provinces of Thailand. Two genotypes (SEA2 and SEA3) were commonly distributed among these provinces. The understanding of the diversity of *O. tsutsugamushi*, especially of the two common genotypes, is important especially in view of future design and evaluation of
sensitive and specific diagnostic assays and vaccine candidates. Previous human trials with scrub typhus vaccine candidates in the field have failed due to the inability of the vaccines to protect against exposure to disparate *O. tsutsugamushi*. Thus, according to their dominance and distribution the genotypes described in this report should be considered for incorporation into future scrub typhus vaccines along with other commonly found isolates causing human disease.
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REFERENCES


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TABLE 1. Representatives of each Orientia tsutsugamushi strain used for phylogenetic characterization
TABLE 2. Evidence of *Orientia tsutsugamushi* infection in blood samples collected from FUO patients’ by different regions of Thailand using indirect fluorescence antibody (IFA) and polymerase chain reaction (PCR) methods

Febrile illness patient samples tested for evidence of *O. tsutsugamushi* infection

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FIGURE LEGENDS

FIG. 1. Phylogenetic trees of *Orientia tsutsugamushi* based on partial 56 kDa TSA gene sequences demonstrating the relationships among human and rodent strains found in Thailand and other listed in GenBank. The tree was midpoint rooted. Bootstrap values >50% were labeled over branches (1,000 replicates). Human and rodent strains described in this report are shown in bold. (A) Neighbor-joining tree: the tree was generated by Kimura two-parameter method with gamma correction (gamma shape parameter = 0.9561). (B) Maximum parsimony tree: the tree was generated using heuristic search with random step-wise addition (10 replicates).

FIG. 2. Maximum-likelihood tree based on partial 56 kDa TSA gene sequences of *Orientia tsutsugamushi* demonstrating the relationships among human and rodent strains found in Thailand and other listed in GenBank. The tree was generated by the step-wise addition method using general time-reversible method with consideration of gamma-distributed rate heterogeneity across sites (−lnL = 6904.15, gamma shape parameter = 0.9561). The tree was midpoint rooted. Bootstrap values >50% were labeled over branches (1,000 replicates). Human and rodent strains described in this report are shown in bold. Thai genotypes are shown by italic bolding. SEA, Southeastern Asia; TH, Thailand; SR, Southern Region; ER, Eastern Region; NR, Northern Region; NER, Northeastern Region.

FIG. 3. Genotype distribution of *O. tsutsugamushi* in six provinces of Thailand.