Characterization Based on the 56-Kda Type-Specific Antigen Gene of *Orientia tsutsugamushi* Genotypes Isolated from *Leptotrombidium* Mites and the Rodent Host Post-Infection

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Abstract. Characterization of the 56-kDa type-specific antigen (TSA) genes of *Orientia tsutsugamushi* (OT) from three naturally infected, laboratory-reared mite colonies comprising three species (*Leptotrombidium deliense* [Ld], *Leptotrombidium imphalum* [Li], and *Leptotrombidium chiangraiensis* [Lc]) has revealed the presence of single and coexisting OT genotypes found in individual chiggers. The Karp genotype was found in all of the chiggers examined, whereas Gilliam and UT302 genotypes were only observed in combination with the Karp genotype. From analysis of these OT genotypes after transmission from chiggers to mice it was determined that with the Lc and Li mites, the OT genotype composition in the rodent spleens post-infection had not changed and therefore resembled that observed in the feeding chiggers. However, only the Karp genotype was found in rodents after feeding by Ld chiggers carrying Karp and Gilliam genotypes. The current findings reveal a complex association among the host, pathogen, and vector.

INTRODUCTION

Orientia tsutsugamushi, the causative agent of scrub typhus, is an obligate intracellular Gram-negative bacterium vectored by larval *Leptotrombidium* mites (chiggers). Mites serve as reservoirs and the bacterium is maintained in successive mite generations by transovarial transmission.^{1–5} The chigger infects a rodent or human host when feeding on tissue fluid.^{6–8} With no vaccine, scrub typhus prevention is a major challenge and infections may be fatal if untreated.⁹ *Orientia tsutsugamushi*-infected mites are widely distributed throughout many Asian countries, northern Australia, and the western Pacific islands thereby placing an estimated one billion people at risk for scrub typhus, which averages one million cases annually.¹⁰

Orientia tsutsugamushi has been characterized through serotyping on the basis of reactivity to hyperimmune serum raised against prototype strains (Karp, Gilliam, Kato, TA716)^{11,12} and phylogenetic analysis using 56-kDa type-specific antigen (TSA) sequences from outer membrane protein-encoding genes.¹³ The 56-kDa TSA of *O. tsutsugamushi* is the immunodominant protein¹⁴ for which the conserved domain regions of the 56-kDa TSA are responsible for cross-reactivity of antisera against diverse serotypes, whereas the variable domains I–IV can be used for serotype identification.¹⁵

A past study showed the most prevalent *O. tsutsugamushi* serotype found in rodents and vectors in Thailand was Karp, whereas other serotypes (Gilliam, Kato, TA716, and TA763) were proportionately less common.¹⁶ However, recent genotyping based on the 56-kDa TSA gene sequence has shown significant diversity in the nucleotide sequence of *O. tsutsugamushi* isolates from patients and rodent hosts in many parts of Thailand. The resulting genotypes exhibited much lower similarity to the prototype strains than previously described.^{17,18} In the current study, we also use the 56-kDa TSA gene sequence (700 basepairs [bp]) to

characterize *O. tsutsugamushi* from 12 naturally infected, laboratory-reared *Leptotrombidium* colonies (comprising three *Leptotrombidium* species: *L. deliense*, *L. imphalum*, and *L. chiangraiensis*) located at the Armed Forces Research Institute of Medical Sciences in Bangkok, Thailand (AFRIMS). As knowledge regarding the transmission dynamics of *O. tsutsugamushi* and chigger-host interactions is limited, we evaluated the degree to which the composition of *O. tsutsugamushi* genotype(s) found in an individual chigger is maintained in the rodent host post-infection. In addition, *O. tsutsugamushi* were characterized by phylogenetic analysis of the 56-kDa TSA gene sequences from field-collected rodents and chiggers to supplement our findings.

MATERIALS AND METHODS

Laboratory-reared *O. tsutsugamushi*-infected mites. Twelve *O. tsutsugamushi*-infected mite colonies (four lines of *L. chiangraiensis*, seven lines of *L. imphalum*, and one line of *L. deliense*) are maintained in an animal biosafety level-3 (ABSL-3) facility at AFRIMS (Table 1).¹⁹ Larval mites were fed the ears of Institute of Cancer Research (ICR) mice from a Charles River Technology (BioLASCO, Taiwan) colony maintained by the Department of Veterinary Medicine, AFRIMS, whereas nymphs and adults were fed a diet of springtail eggs (protocol #09-11, Maintenance of the *Leptotrombidium* larval mite colonies: chigger feeding on ICR mice [*Mus musculus*]).

Extraction of genomic DNA from *O. tsutsugamushi* -infected mites. *Orientia tsutsugamushi*-infected chiggers from different lines of *Leptotrombidium* spp. were individually subjected to genomic DNA extraction using a modified tissue protocol from the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). An infected chigger was placed in a 1.5-mL microcentrifuge tube and punctured with a fine needle. Ninety microliters of ATL lysis buffer was added and the sample was either processed immediately or stored at –70°C. Ten microliters of Proteinase K solution (20 mg/mL) was added and the sample was incubated at 56°C for 3 h. Subsequently, 100 µL of AL buffer was added and the sample

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TABLE 1

Origins of 12 *Orientia tsutsugamushi*-infected *Leptotrombidium* mite colonies maintained at Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand

Colony	Date established	Mite species	Host	Location
Ld-1	9 Sep 1992	<i>L. deliense</i>	<i>Tupaia glis</i>	Bangkruai, Nonthaburi
Li-1	24 Sep 1993	<i>L. imphalum</i>	<i>Rattus koratensis</i>	Ban Thungpha, Chiang Rai
Li-2	25 Nov 1993	<i>L. imphalum</i>	<i>Rattus rattus</i>	Ban Pagook, Chiang Rai
Li-3	25 Nov 1993	<i>L. imphalum</i>	<i>Rattus rattus</i>	Ban Pagook, Chiang Rai
Li-4	18 May 1994	<i>L. imphalum</i>	<i>Rattus rattus</i>	Ban Nongkrok, Chiang Rai
Li-5	19 Oct 1995	<i>L. imphalum</i>	<i>Rattus rattus</i>	Ban Maesad, Chiang Rai
Li-6	19 Jun 1996	<i>L. imphalum</i>	<i>Rattus rattus</i>	Ban Nongkrok, Chiang Rai
Li-7	19 Jun 1996	<i>L. imphalum</i>	<i>Rattus rattus</i>	Ban Nongkrok, Chiang Rai
Lc-1	13 Jan 1994	<i>L. chiangraiensis</i>	<i>Rattus losea</i>	Ban Pagook, Chiang Rai
Lc-2	18 Oct 1995	<i>L. chiangraiensis</i>	<i>Rattus rattus</i>	Ban Pagook, Chiang Rai
Lc-4	15 Jul 1998	<i>L. chiangraiensis</i>	<i>Rattus rattus</i>	Ban Pagook, Chiang Rai
Lc-5	15 Jul 1998	<i>L. chiangraiensis</i>	<i>Rattus rattus</i>	Ban Pagook, Chiang Rai

mixed by pulse-vortexing for 15 s followed by incubation at 70°C for 10 min. One hundred microliters of absolute ethanol was added and the sample mixed by pulse-vortexing for 15 s. The sample was then applied to a QIAamp spin column and DNA was eluted in 50 µL AE buffer and stored at -20°C until amplification.

Amplification and sequencing of the 56-kDa TSA. The 56-kDa TSA gene was amplified by nested polymerase chain reaction (nPCR) using previously described primers²⁰ and in-house primers designed from published sequences (GenBank accession nos. AY956315, M33004). The first amplification step was performed using in-house designed primers; CG56F (5'-TTA CAA TGG ATA AAA CGC TTT GAA-3') and CG56R (5'-AGA AAA ACC TAG AAG TTA TAG CGT ACA-3') and the second step used previously described primers RTS8 and RTS9.²⁰ A volume of 2.5 µL of extracted DNA was used as template in the first amplification step. One-tenth of the total volume from the first amplification was used for the second amplification. A total of 25 µL of the PCR reaction mixture contained template DNA, 400 nM of each primer, 200 µM of dNTP, 1× PCR buffer, and 0.5 units of iProof High-Fidelity DNA polymerase (Bio-Rad, Hercules, CA). Amplification was performed using a DNA thermal cycler (GeneAmp PCR System 2700, Applied Biosystems, Foster City, CA) under the following conditions: initial denaturation at 98°C for 2 min; 40 cycles of 98°C for 10 s (denaturation); 53°C for 20 s (annealing); 72°C for 1 min (extension); and 72°C for 5 min. The second amplification step was carried out following the same protocol above except that the annealing step in the cycle was 55°C for 15 s and the extension period was shortened to 45 s. The PCR product was run on agarose gel (1.5%) using electrophoresis and visualized with GelStar Nucleic Acid Gel Stain (Lonza, Basel, Switzerland). The image of the gel was documented using a Gel imaging system-U:Genius (Syngene, Cambridge, UK).

Characterization of *O. tsutsugamushi* genotype. The PCR product of 56-kDa TSA gene (700 bp) was purified by Qiagen DNA purification kit (Qiagen) for removal of salts and primer dimers. Purified PCR fragment of about 700 bp was then cloned into pCR2.1-TOPO vector (Invitrogen, Foster City, CA) and grown in *Escherichia coli* (DH5α-T1^R). Seventeen to 20 *E. coli* colonies carrying a plasmid successfully harboring 56-kDa insert were randomly selected for sequencing. Plasmids were purified from *E. coli* using QIAprep Spin Miniprep Kit

(Qiagen) and sent for DNA sequencing (Sanger method) at AITBiotech (Singapore).

Transmission of *O. tsutsugamushi* from the chigger to the rodent host. A single chigger from each of three *O. tsutsugamushi*-infected chigger lines (*L. chiangraiensis* [Lc-1], *L. imphalum* [Li-1], and *L. deliense* [Ld-1]) was individually placed on the ear of an anesthetized *O. tsutsugamushi*-naive ICR mouse (4 to 10 weeks of age). Mice were restrained in cages for 3 to 4 d, removed, and then transferred to a polycarbonate shoebox cage. The chigger was removed from each mouse, placed in a 1.5-mL tube, and punctured with a fine needle. Ninety microliters of ATL lysis buffer (Qiagen) were added and the sample either processed immediately or stored at -70°C. Mice were clinically observed for 30 d for signs and symptoms of *O. tsutsugamushi* infection. Mice infected with *O. tsutsugamushi* were euthanized when they exhibited signs of moribund illness. The liver, spleen, and kidneys were dissected and stored at -70°C. Mice not developing signs of clinical scrub typhus infection at the end of the 28-d observation period were euthanized, and blood and organs collected for determination of *O. tsutsugamushi* presence using nPCR.²⁰ The procedures described previously were approved by the AFRIMS Institutional Animal Care and Use Committee (IACUC) under protocol number 09-11 entitled, "Maintenance of the *Leptotrombidium* larval mite colonies: chigger feeding on ICR mice (*Mus musculus*)."

Extraction of genomic DNA from infected mice. Extraction of genomic DNA from the spleens of infected mice obtained from the transmission experiment described previously was performed using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Samples of infected spleen were placed into a 1.5-mL tube containing 600 µL of chilled nucleic lysis solution and incubated at 65°C for 15–30 min. Proteinase K solution (20 mg/mL) was added to reach a final concentration of 580 µg/mL and the solution incubated at 55°C for 3 h. Three microliters of RNase solution were added and the mixture incubated at 37°C for 15–30 min. Undesirable protein was precipitated out by first adding 200 µL of protein precipitation solution. The solution was vortexed, placed on ice for 5 min, and spun at 13,000 rpm for 4 min at room temperature. The supernatant was transferred to a sterile tube and 600 µL of isopropanol was added to precipitate the DNA. The DNA was recovered by centrifugation at 13,000 rpm for 1 min at room temperature, briefly washed with 70% ethanol, air-dried, and resuspended in 100 µL of DNA rehydration

solution. The PCR, product purification, and cloning were carried out as described previously.

Field-collected rodents and chiggers. Rodent and chigger collections were conducted during the wet season (August 2010) in Buri Ram and Chachoeng Sao provinces, Thailand. Field-collected rodents were identified to species and the blood obtained by cardiac puncture and organs collected following dissection as previously described.²¹ Chiggers were removed from the ears and stored in 70% ethanol. Animal collection procedures were approved by the AFRIMS IACUC under protocol number 09-02 entitled, "Field sampling of small mammal populations to support zoonotic disease surveillance and ectoparasite collection."

Quantitative real-time PCR (qPCR) screening for *O. tsutsugamushi* from field-collected rodents/chiggers. Field-collected rodents and chiggers were screened for *O. tsutsugamushi* by a qPCR assay.²² The primers and probe were designed to detect a portion of the 47-kDa HtrA gene. The reaction mixture (25 μ L) contained 12.5 μ L 2 $\times$ Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 0.2 μ M probe, and 0.2 μ M each of primers. The qPCR assay, Otsu47, was performed by incubating the sample at 95°C for 2 min followed by 45 cycles of 95°C for 15 s, and 60°C for 1 min using a chromo4 Real-time PCR detector (Bio-Rad). Rodents or chiggers found to be positive were further characterized by amplification and sequencing of the 56-kDa TSA as described previously.

Sequence and phylogenetic analysis. Sequences were edited and assembled using Sequencer program v4.1.4 (Applied Biosystems) and compared with sequences in the BLASTN database (National Center for Biotechnology Information). Multiple sequence alignment was performed using the CLUSTALW program.²³ The Neighbor-Joining (NJ) tree with bootstrapping (2,000 replicates) was constructed using the *p*-distance model of nucleotide substitution (56-kDa gene variable domains I-III) in the MEGA5 program.²⁴

Determination of serological reactivity to *O. tsutsugamushi* 56-KDa recombinant proteins by enzyme-linked immunosorbent assay (ELISA). The experiment was performed as previously described.²⁵ The ELISA plates were coated

with 0.3 μ g/well/100 μ L of Karp, Gilliam, Kato, and TA763 56-KDa recombinant protein overnight at 4°C. Briefly, coated plates were first rinsed three times with 1 $\times$ phosphate-buffered saline (PBS) containing 0.1% Tween 20 (1 $\times$ PBST), and then blocked with 200 μ L/well of 10% milk in 1 $\times$ PBST, and incubated for 1 h at room temperature. Mouse serum was serially diluted by a factor of 4 (1:100, 1:400, 1:1,600) in 5% milk in 1 $\times$ PBST. Plates were washed after blocking and incubated with diluted mouse serum for 1 h at room temperature. The plates were then washed again and incubated with Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:4,000 dilution for 1 h at room temperature. At the end of incubation, plates were washed and substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added. The plates were incubated at room temperature for 15–30 minutes in the dark and then read the optical density (OD) at 405–650 nm on an UVmax kinetic microplate reader (Molecular Devices, Sunnyvale, CA). The mean of the negative controls plus two times the standard deviation was used as the cutoff value for determining a positive result. The cutoff value was 0.1 for mouse serum samples.

Animal care and use. The experiments reported herein were conducted in compliance with the Animal Welfare Act and in accordance with the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 1996.

RESULTS

Characterization of *O. tsutsugamushi* from colonized *Leptotrombidium* lines. The full-length 56-kDa TSA DNA gene (1.8 kb) and ~700-bp nested PCR product covering variable domains I-III were amplified from individual chigger from each of 12 distinct *Leptotrombidium* lines maintained in colony at AFRIMS (Figure 1A and B). We selected one line from each *Leptotrombidium* species (Lc-1, Li-1, Ld-1) for further characterization of *O. tsutsugamushi*

FIGURE 1. Polymerase chain reaction (PCR) products derived from the amplification of (A) the full-length and (B) variable domains I-III 56-kDa TSA gene of *O. tsutsugamushi* from 12 infected *Leptotrombidium* colonies.

TABLE 2

Sequence distance of 56-kDa TSA gene sequences (Variable Domains I–III, 640 bp) of three *Orientia tsutsugamushi*-infected *Leptotrombidium* mite colonies (Lc-1, Li-1, Ld-1), references *O. tsutsugamushi* Karp, Gilliam, Kato, TA763, and other Genotypes

	Lc-1	Li-1a	Li-1b	Ld-1a	Ld-1b	Karp	Gilliam	Kato	TA763	UT302	Taitung4
Lc-1 (19*)	***	81.3	96.9	83.2	97.5	94.2	84.3	81.2	83.8	82.1	81.0
Li-1a (15/20*)	21.6	***	79.9	76.5	80.3	78.0	79.2	77.1	85.5	98.5	99.1
Li-1b (5/20*)	3.2	23.5	***	82.5	95.4	93.2	83.3	80.3	83.8	80.6	79.6
Ld-1a (11/17*)	19.1	28.3	19.9	***	20.1	82.1	93.0	79.8	80.4	77.2	76.2
Ld-1b (6/17*)	2.6	22.9	4.7	82.4	***	93.2	84.5	79.6	82.6	81.6	79.9
Karp	6.1	26.1	7.2	20.5	7.1	***	83.7	77.8	81.9	78.6	78.0
Gilliam	17.7	24.5	18.9	7.5	17.3	18.4	***	79.8	82.7	78.9	79.0
Kato	21.8	27.4	23.0	23.7	24.0	26.5	23.7	***	78.4	77.4	76.6
TA763	18.3	16.2	18.3	22.7	19.9	20.7	19.8	25.7	***	85.9	85.1
UT302	20.5	1.5	22.5	27.2	21.2	25.3	24.8	27.0	15.7	***	2.2
Taitung4	22.0	0.9	23.9	28.7	23.5	26.1	24.7	28.3	16.7	97.9	***

Percent similarity shows in upper triangle and percent divergence in lower triangle. Letter “a” and “b” indicate the difference of sequence type. Highest identity with reference genotypes are shown in bold type.

*Number of bacterial colonies with a 56-kDa type-specific antigen (TSA) insert, matching reference genotype per total number of colonies evaluated.

strains/genotypes maintained by that species. Sequence analysis of the partial 56-kDa gene (700 bp) of *O. tsutsugamushi* from 17 to 20 sequences/clones randomly selected from each chigger (*Leptotrombidium*) species showed the presence of *O. tsutsugamushi* genotypes closely related to Karp, UT302, or Gilliam (Table 2, Figure 2). Alignment of 19 sequences of the 56-kDa genes selected from one Lc-1 resulted in one identical genotype closely related to Karp-related genotypes with 94.2% similarity to the reference Karp genotype

(Table 2, Figure 2). However, the alignment of 56-kDa TSA sequences derived from one Li-1 and from one other Ld-1 chigger revealed different results.

The alignment of 20 sequences from Li-1 and 17 sequences from Ld-1 lines resulted in two distinct genotypes found in single chigger, designated “a” and “b” in both lines. For the Li-1 line, the majority of sequences (15 of 20 sequences) clustered with the UT302-related genotypes consisting of TA763, TA716, Taitung-4, UT302, and CB19 with 99.1%

FIGURE 2. Neighbor-joining (NJ) tree derived from partial 56-kDa TSA genes (variable domains I–III, 498 bp) of *O. tsutsugamushi*. The analysis involved 46 nucleotide sequences of *O. tsutsugamushi*-infected chigger lines (Lc-1, Li-1, Ld-1), *O. tsutsugamushi*-infected wild-caught rodent and chigger along with reference sequences retrieved from the GenBank database as shown in Table 5. The NJ tree was constructed using the *p*-distance method of nucleotide substitution model. The bootstrap *P* values > 50% are indicated at major nodes (2,000 replicates). Tree is rooted at its midpoint. Scale bar represents substitutions per site.

and 98.5% sequence similarity to genotypes Taitung-4 and UT302, respectively (Figure 2, Table 2). This sequence type was designated as Li-1a. The remaining five sequences from the Li-1 line (designated Li-1b) grouped with Karp-related genotypes with 93.2% sequence similarity to the reference Karp and 98.8% similarity to a nearby cluster of genotypes that included UT76 and UT332 (Figure 2). For the Ld-1 line, 11 of 17 sequences were identical and clustered with the Gilliam group (designated Ld-1a) with 93.0% sequence similarity to the reference Gilliam and 97.8% sequence similarity to a nearby group of genotypes that included UT125 and UT196 (Figure 2). The remaining six sequences (designated Ld-1b) fell into Karp-related genotypes with 93.2% sequence similarity to reference Karp and 98.0% to the TW45R genotype. The sequence similarity among Karp-related genotypes found in Lc-1, Li-1b, and Ld-1b was relatively high (95.4% to 97.5%). Although Lc-1 and Ld-1b grouped together in the same cluster, Li-1b formed a separate group distant from but shared common ancestor with Lc-1/Ld-1b types.

***O. tsutsugamushi* genotype characterization from laboratory-reared rodents and chiggers post-infection.** The previous results show that Lc-1, Li-1, and Ld-1 lines maintain *O. tsutsugamushi* Karp-related, Karp-related/UT302, and Karp-related/Gilliam genotypes, respectively. We determined whether the composition of *O. tsutsugamushi* genotype(s) found in the rodent post-infection reflected the pattern observed in the chigger that fed on the rodent. Mice challenged with Lc-1 and Li-1 showed signs of illness within 10 d post-infection, whereas the mouse challenged with Ld-1 did not develop signs of infection within the 28-d period of observation, although spleen enlargement and *O. tsutsugamushi*-specific antibodies were observed (data not shown). Spleens from mice challenged with Lc-1, Li-1, and Ld-1 were positive for *O. tsutsugamushi* as determined by nPCR assay. Analysis of 56-kDa sequences showed that the composition of *O. tsutsugamushi* genotype(s) in spleens from infected mice generally mimicked the pattern observed in the Lc-1 and Li-1 chiggers, which fed on the mice (Table 3). Twenty sequences from the Lc-1 chigger that fed on an *O. tsutsugamushi*-naïve rodent were found

to be a member of the Karp-related genotype (94.2% similarity) and this same pattern was observed in the rodent spleen post-transmission (Table 3). The sequence alignment shows almost no variability in sequences within and between the chigger and the rodent (data not shown). The UT302-related sequences (98.5% similarity) comprised the majority of sequences (12 of 18) in Li-1 and a similar majority (17 of 19) was maintained in the rodent post-infection (98.5% similarity) (Table 3). Karp-related genotype sequences comprised a minority of sequences in the Li-1 chigger (6 of 18; 5 with 93.2% similarity and 1 with 99.8% similarity) and a similar minority was maintained in the rodent (2 of 19 with 93.2% similarity) post-infection. However, the pattern exhibited in the Ld-1 chigger differed from that observed in the rodent spleen. Even though Karp-related (7 of 18 sequences with 93.2% similarity) and Gilliam (11 of 18 sequences with 93.0% similarity) genotypes were found in the Ld-1 chigger, only the Karp-related genotype (20 of 20 sequences with 93.2% similarity) was found in the mouse spleen post-infection.

***O. tsutsugamushi* genotype characterization from field-collected rodents and chiggers.** The data of *O. tsutsugamushi* characterization reported previously are solely from the experiments using laboratory-reared *Leptotrombidium* chiggers, even though the original mite colonies were established from chiggers collected from wild-caught rodents in Thailand, however, the mite colonies have been maintained in a laboratory environment for over 10 years. To confirm the coexistence of *O. tsutsugamushi* genotypes in wild chigger and rodent populations, we examined chiggers and rodents collected from field sites in Thailand during the rodent-borne diseases surveillance in 2010. One *O. tsutsugamushi*-positive rodent from each province was selected for 56-kDa TSA sequence analysis. *Bandicota indica* from Buri Ram (BA0185) contained 14 of 20 Karp-related genotype sequences that were most similar to Lc-1 (Table 4, Figure 2). Five of 20 sequences were Gilliam-related genotype sequences most similar to Ld-1a. Finally, 1 of 20 sequences was most similar to the UT302-related genotype particularly to Taitung-4 and Li-1a. No chiggers from BA0185 (total $N = 16$ chiggers) were positive for *O. tsutsugamushi*. The rodent captured in Chachoeng Sao (BA0344) contained only Gilliam-related genotype sequences (15 of 15 sequences) that were most similar to UT125, UT196, and UT144. Two of 7 chiggers from this same rodent were positive for *O. tsutsugamushi*. Characterization of one of the two chiggers showed coexistence of Gilliam-related (4 of 18 sequences) and UT302-related (14 of 18 sequences) genotypes. The Gilliam-related genotype sequences were most similar to Ld-1a and the UT302-related genotype sequences were most similar to Taitung-4 and Li-1a.

DISCUSSION

Characterization of *O. tsutsugamushi* based on the 56-kDa TSA gene sequence revealed the coexistence of genotypes (Karp/Gilliam or Karp/UT302) in naturally infected, laboratory-reared mites (*L. imphalum* and *L. deliense*) and in one field-collected rodent (Gilliam/Karp/UT302) and chigger, *Leptotrombidium* spp. (Gilliam/UT302). Further evaluation revealed that the composition of *O. tsutsugamushi*

TABLE 3

Similarities of 56-kDa TSA gene sequences of the variable domains I–III from chiggers (Lc-1, Li-1, Ld-1) and the rodent post-infection to reference genotypes (Karp, Gilliam, UT302)

Mouse feeding experiment	Sample	Sequence type	Sequence similarity to reference genotypes*		
			Karp (AY956315)	UT302 (EF213095)	Gilliam (DQ485289)
Lc-1	Chigger	Lc-1	20/20 (94.2)	–	–
	Mouse spleen	Lc-1	20/20 (94.2)	–	–
Li-1	Chigger	Li-1a	–	12/18 (98.5)	–
		Li-1b	5/18 (93.2)	–	–
		N/A	1/18 (99.8)	–	–
	Mouse spleen	Li-1a	–	17/19 (98.5)	–
Ld-1	Chigger	Li-1b	2/19 (93.2)	–	–
		Ld-1a	–	–	11/18 (93.0)
	Mouse spleen	Ld-1b	7/18 (93.2)	–	–
		Ld-1b	20/20 (93.2)	–	–

*Number of bacterial colonies with a 56 kDa type-specific antigen (TSA) gene insert matching reference genotype/total number of colonies evaluated. Numbers in parentheses represent percent sequence similarity to corresponding reference genotype.

TABLE 4

Characterization of *Orientia tsutsugamushi* obtained from field-collected rodents and a chigger in Thailand based on 56-kDa TSA sequence homology to reference genotypes (Karp, Gilliam, UT302)

Location	Rodent species	No. of infected chiggers/total collected*	Similarity to reference genotypes (no. of 56-kDa TSA sequences matching reference genotype/total sequences analyzed)†	
			Rodent	Chigger‡
Buri Ram (Forest)	<i>Bandicota indica</i> (BA0185)	0/16	Gilliam (5/20)	–
			Karp (14/20)	–
			UT302 (1/20)	–
			Gilliam (15/15)	Gilliam (4/18)
Chachoeng Sao (Rice field)	<i>Bandicota indica</i> (BA0344)	2/7‡		UT302 (14/18)

*Determined by Otsu47 quantitative real-time polymerase chain reaction (qPCR) assay on DNA sample extracted from an individual chigger.

†Number of bacterial colonies with a 56-kDa type-specific antigen (TSA) insert matching reference genotype/total number of colonies evaluated.

‡Characterization performed on one infected chigger.

genotype(s) observed in an individual chigger during feeding on an *O. tsutsugamushi*-naïve rodent does not always mimic that which is found in the rodent post-infection. As such, these findings show a complex association between the host, pathogen, and vector.

Our results show isolates genetically related to members of the Karp genotype based on the 56-kDa TSA to be the most prevalent *O. tsutsugamushi* among the naturally infected mites maintained in our ABSL-3 laboratory. Each *L. chiangraiensis* line contained sequences belonging to Karp-related genotype, whereas *L. imphalum* and *L. deliense* each contained a member of the Karp genotype in coexistence with a member of the UT302 and Gilliam genotypes, respectively. These latter findings are consistent with our

results from field-collected rodents and a chigger, and complement a previous report, which showed polytypic infections of *O. tsutsugamushi* in patient samples from Udon Thani province, Thailand using multi-locus sequence typing (MLST).²⁶ Additionally, although the antigenic combination of *O. tsutsugamushi* strains has been previously observed in chiggers^{27,28} and human isolates from Malaysia using direct immunofluorescence staining and the complement fixation test,²⁹ cross-reactivity was observed for type-specific monoclonal antibodies (mAbs) that recognize the 56-kDa antigen.³⁰ Indeed, it is widely assumed that the 56-kDa TSA undergoes strong selection pressure from the host immune response and therefore the protein sequences change rapidly for bacteria to escape the immune response, resulting in antigenic diversity and the creation of new immunotypes based on the 56-kDa TSA gene. Therefore, 56-kDa TSA gene sequencing or MLST are preferable approaches to characterize *O. tsutsugamushi* isolates. In this study, it was possible to test only a single chigger from each line because of logistical considerations; however, future studies will be performed to show if most of any given hatch would yield identical rickettsial strain patterns.

To our knowledge, this is the first report to characterize *O. tsutsugamushi* genotype transmission from the chigger to the rodent host. In the case of Lc-1 and Li-1, the composition of genotypes found in the mouse spleen 14 d post-feeding was the same as that observed in the chigger during feeding. However, only the Karp genotype was found in the mouse that was fed upon by an Ld-1 chigger that carried multiple genotypes (Gilliam and Karp genotypes). The mechanism responsible for this latter observation is unknown. Interestingly, the Ld-1-infected mouse did not develop signs of illness and the antibody titer to the Karp prototype antigen, determined by ELISA assay, was found to be very high, whereas mice fed upon by either Lc-1 or Li-1 chiggers developed symptoms 10 d post-feeding with antibody titers lower than those observed in the mouse associated with the Ld-1 chigger (data not shown).

We clearly show significant variability in the 56-kDa gene of *O. tsutsugamushi* among chiggers. However, analysis of nucleotide sequences from the complete ORF 47-kDa gene from our 12 infected lines found no significant differences in the alignment of 47-kDa sequences among bacteria from *O. tsutsugamushi*-infected chiggers and those from reference genotypes (bootstrap *P* value ≤ 25) with sequence similarities ranging from 96.9% to 99.4% (data not shown). Such data, similar to recently described limited sequence variation of the 47-kDa gene found among recent human

TABLE 5

Descriptions of *Orientia tsutsugamushi* isolates and reference genotypes used in this study

Isolate	Source	Country	Accession no.
CB19	<i>Rattus rattus</i>	Thailand	GU068058
CB62	<i>Bandicota indica</i>	Thailand	GU068055
FPW2016	Human	Thailand	EF213085
Gilliam	Human	Japan	DQ485289
Hirahata	<i>Leptotrombidium pallidum</i>	Taiwan	AF201835
Je-cheon	Human	Taiwan	AF430143
Karp	Human	Taiwan	AY956315
Kato	Human	Japan	AY836148
Kawasaki	Human	Japan	M63383
Kuroki	–	Japan	M63380
LA-1	<i>Leptotrombidium arenicola</i>	Japan	AF173049
LF-1	–	Japan	AF173050
Shimokoshi	–	Thailand	M63381
TA678	<i>Rattus rattus</i>	Thailand	U19904
TA686	<i>Tupaia glis</i>	Thailand	U80635
TA716	<i>Menetes berdmorei</i>	Thailand	U19905
TA763	<i>Rattus rajah</i>	Taiwan	U80636
Taitung-4	–	Taiwan	AY787232
TW45R	<i>Rattus losea</i>	Taiwan	AY222632
TW461	<i>Rattus rattus</i>	Taiwan	AY222631
TWyu81	<i>Leptotrombidium pallidum</i>	Thailand	AY222640
UT176	Human	Thailand	EF213078
UT125	Human	Thailand	EF213096
UT144	Human	Thailand	EF213091
UT167	Human	Thailand	EF213080
UT176	Human	Thailand	EF213081
UT177	Human	Thailand	EF213084
UT196	Human	Thailand	EF213079
UT219	Human	Thailand	EF213100
UT302	Human	Thailand	EF213095
UT332	Human	Thailand	EF213083
Yeojo	Human	South Korea	AF430144
Yonchon	–	South Korea	U19903
Young-world	Human	South Korea	AF430141

O. tsutsugamushi isolates and reference strains,³¹ provide justification for using the 47-kDa protein as an immunogen in the development of a scrub typhus vaccine as has been suggested previously.³²

The host response observed after feeding by *L. deliense* in which the Gilliam genotype was not transmitted to the host, provides an impetus to better characterize the immune response of the host to infection. Our results highlight the need to better understand the transmission dynamics of *O. tsutsugamushi*, chigger-host interactions, and the host immune response in an effort to develop an effective vaccine against scrub typhus.

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