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Diversity of the 47-kD HtrA Nucleic Acid and Translated Amino Acid Sequences from 17 Recent Human Isolates of *Orientia*

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Abstract

Orientia tsutsugamushi, the etiologic agent of potentially fatal scrub typhus, is characterized by a high antigenic diversity, which complicates the development of a broadly protective vaccine. Efficacy studies in murine and nonhuman primate models demonstrated the DNA vaccine candidate pKarp47, based upon the *O. tsutsugamushi* Karp 47-kD HtrA protein gene, to be a successful immunoprophylactic against scrub typhus. To characterize 47-kD HtrA protein diversity among human isolates of *Orientia*, we sequenced the full open reading frame (ORF) of the 47-kD HtrA gene and analyzed the translated amino acid sequences of 17 patient isolates from Thailand ($n=13$), Laos ($n=2$), Australia ($n=1$), and the United Arab Emirates (UAE) ($n=1$) and 9 reference strains: Karp (New Guinea), Kato (Japan), Ikeda (Japan), Gilliam (Burma), Boryong (Korea), TA763, TH1811 and TH1817 (Thailand), and MAK243 (China). The percentage identity (similarity) of translated amino acid sequences between 16 new isolates and 9 reference strains of *O. tsutsugamushi* ranged from 96.4% to 100% (97.4% to 100%). However, inclusion of the recently identified *Orientia chuto* sp. nov. reduced identity (similarity) values to 82.2% to 83.3% (90.4% to 91.4%). These results demonstrate the diversity of *Orientia* 47-kD HtrA among isolates encountered by humans and therefore provide support for the necessity of developing a broadly protective scrub typhus vaccine that takes this diversity into account.

Key Words: *Orientia tsutsugamushi*—Scrub typhus—47-kD HtrA protein gene—Antigenic diversity—vaccine development.

Introduction

Scrub typhus is an acute, febrile, and potentially fatal disease caused by infection with the obligate intracellular bacteria of the genus *Orientia*. It can be characterized by fever, rash, eschar, pneumonitis, meningitis, and coagulopathies, in some cases leading to circulatory collapse (Kawamura et al. 1995). This disease is commonly seen in the Asia-Pacific region, with high population density and an estimated annual 1 million cases of scrub typhus (Rosenberg 1997, Kelly et al. 2002). Furthermore, it has become an identified risk to the increasing number of people traveling to this region (Jensenius et al. 2004, Jensenius et al. 2009). Scrub typhus can account for up to 23% of all febrile episodes and 27% of blood culture–negative fever patients in endemic areas (Brown et al. 1976, Phongmany et al. 2006). Mortality rates for scrub typhus range from <1% to 50%, depending on proper and timely antibiotic treatment, status of the individual infected, and the strain of *Orientia* encountered (Kelly et al. 2002). Recent reports of scrub typhus outbreaks (Kelly et al. 2002) and the recent detection of cases of scrub typhus outside previously described endemic regions (Izzard et al. 2010, Balcells et al. 2011) indicate that the disease is emerging and reemerging, and emphasize the need for characterizing antigen targets

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toward the development of a broadly protective scrub typhus vaccine.

The genus *Orientia*, is a member of the order Rickettsiales, which contains many other arthropod-vectored pathogens (*e.g.*, *Rickettsia, Anaplasma, Ehrlichia*). Until very recently, it consisted of a single species, *Orienta tsutsugamushi*, with multiple antigenically disparate isolates characterized over the past 60 plus years. Initially, 3 antigenically distinct strains of *O. tsutsugamushi* were described: Karp (New Guinea), Kato (Japan), and Gilliam (Burma) (Kelly et al. 2009). In addition to these prototype strains, more than 20 antigenically distinct serotypes have been recognized. In addition to the various antigenic types, at least 9 phylogenetic groups include 135 isolates of *O. tsutsugamushi* based upon the extremely variable 56-kD type-specific antigen (TSA) gene have been described (Kelly et al. 2009).

Subunit vaccines based on the 47-kD high-temperature requirement A (HtrA) and 56-kD TSA proteins have been developed and evaluated (Chattopadhyay et al. 2007). Recombinant protein (truncated) and plasmid DNA 56-kD TSA vaccine candidates have shown the ability to provide mice with homologous protection (Ni et al. 2005, Chattopadhyay et al. 2007). Due to the type-specific nature of this protein, the more conserved antigen 47-kD HtrA protein (Oaks et al. 1989, Moree et al. 1992) was evaluated with the purpose of developing a vaccine with broad protection. This protein is a member of the HtrA protein family, which is characterized by serine protease and chaperone activities. The 47-kD HtrA gene and gene product of the Karp strain have been used to develop DNA (Niu et al. 2003, Xu et al. 2005) and recombinant protein (Niu et al. 2003, Yu et al. 2005) vaccine candidates displaying immunogenicity and efficacy in mouse scrub typhus models. Recent evaluations in our laboratory have shown not only that the pKarp47 DNA vaccine candidate is highly effective in protecting CD-1 outbred mice from homologous challenge, but also in protecting them from heterologous challenge from 4 of 17 disparate isolates using a single immunization (A.L.R., unpublished data). Most recently, the pKarp47 vaccine candidate was successful in completely protecting 2 and partially protecting 3 of 5 cynomolgus monkeys from intradermal injection with homologous challenge (D.H. Paris et al., manuscript in preparation).

With the intention of developing a broadly protective scrub typhus vaccine incorporating the 47-kD HtrA target, we investigated the diversity of this gene from recently acquired human clinical isolates of *Orientia*. We report here our success in determining the nucleic acid and translated amino acid sequence diversity based on the full open reading frame (ORF) of the 47-kD HtrA gene of 17 newly obtained *Orientia* isolates from Thailand, Laos, Australia, and the United Arab Emirates (UAE), and 9 reference strains from New Guinea, Japan, Burma, Korea, Thailand, and China.

Materials and Methods

Orientia *isolates and nucleic acid preparations*

Blood samples from patients with scrub typhus (clinically diagnosed and subsequently confirmed by serology) were collected at various study sites by the Mahidol–Oxford Tropical Medicine Research Unit, Mahidol University, Bangkok, Thailand (MORU), the Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit, Mahosot Hospital, Vientiane, Laos (LOMWRU), and the Australian Rickettsial Reference Laboratory/Barwon Biomedical Research, the Geelong Hospital, Geelong, Victoria, Australia during 2003–2009. The site of blood collection and therefore georeferencing may not have been the site where the patient was bitten. The blood samples were inoculated into Vero cells and cultured at 37°C to obtain *Orientia* isolates (Luksameetanasan et al. 2007, Izzard et al. 2010). Nucleic acid was extracted from the cultures using the Wizard SV Genomic DNA purification system (Promega, USA) or patient blood samples using Qiagen Mini Blood kit (Qiagen, Valencia, CA). Seventeen dried nucleic acid preparations of *Orientia* isolates, 7 from cultures and 10 from patient blood samples (culture negative), were sent to Naval Medical Research Center (NMRC), Silver Spring, MD, where the nucleic acid preparations were reconstituted in TE buffer. Of the 17 DNA samples, 6 were from Udorn Thani province in northeastern Thailand, 2 from Tak province in northwestern Thailand (Luksameetanasan et al. 2007, Blacksell et al. 2008), 5 from Chiang Rai province in northern Thailand (this report), 2 from Laos (Phongmany et al. 2006), 1 from Australia (Unsworth et al. 2007), and 1 from the UAE (Izzard et al. 2010). The *O. tsutsugamushi* TA763, TH1811, and TH1817 (Thailand) and MAK243 (China) isolates' nucleic acids (NMRC preparations) were analyzed for comparison with the new isolates.

Quantitative real-time PCR (qPCR) assay

The Otsu47 qPCR assay was used to confirm the identity and presence of *Orientia* in the nucleic acid preparations of the new isolates. One microliter of the nucleic acid preparations was added to a final volume of a 25-μL reaction that included Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA). The concentration of primers and probe used and reaction parameters were the same as previously described (Jiang et al. 2004).

PCR, nested PCR, and sequencing

Standard PCR and nested PCR (nPCR) were performed to amplify the entire ORF of the 47-kD HtrA genes. Primers used for PCR (Ot-145F, Ot-1780R) and nPCR (Ot-263F, Ot-1133R, and Otr47-1404R) amplification were selected from conserved regions of the 47-kD HtrA gene after alignment of the sequences from Karp, Kato, Ikeda, Gilliam, and

Table 1. Primers Used for Standard PCR, Nested PCR, and Sequencing of the 47-kD HtrA Protein Gene of Orientia Isolates

Name	*Sequence (5′-3′)*
Ot-145F[a,.b]	ACAGGCCAAGATATTGGAAG
Ot-1780R[a,b]	AATCGCCTTTAAACTAGATTTACTTATTA
OtsuFP630[b]	AACTGATTTTATTCAAACTAATGCTGCT
OtsuRP747[b]	TATGCCTGAGTAAGATACRTGAATRGAATT
Ot-263F[b,c]	GTGCTAAGAAARGATGATACTTC
Ot-1133R[b,c]	ACATTTAACATACCACGACGAAT
Chur 627F[a]	GCGGGATATAGGTAGTTCAA
Chur 669F[b,c]	TATTCAAACTAATGCTGTTGTGC
Otr47-1404R[b,c]	GATTTACTTATTAATRTTAGGTAAAGCAATGT

[a]PCR primer.
[b]Sequencing primer.
[c]Nested PCR primer.

Table 2. Reference Strains and New Isolates of *Orientia* Used in the Study

No.	*Isolate*	*GenBank no.*	*Geographic location*	*Region/Province*	*District/Village*	*Latitude*	*Longitude*	*Ct of Otsu47 qPCR*	*ORF Length*
1	CRF27	HM156047	Thailand N	Chiang Rai	Thoeng	19.683333	100.2	38.28[a]	1401
2	CRF79	HM156048	Thailand N	Chiang Rai	Muang	19.95234	99.88293	44.27[a]	1401
3	CRF93	HM156049	Thailand N	Chiang Rai	Fang	19.91667	99.21667	41.56[a]	1401
4	CRF116	HM156050	Thailand N	Chiang Rai	Muang	19.95234	99.88293	40.66[a]	1401
5	CRF136	HM156051	Thailand N	Chiang Rai	Muang	19.95234	99.88293	34.99[a]	1401
6	FPW1038	HM156052	Thailand NW	Tak	Mae Ramat	16.96667	98.51667	23.61[b]	1404
7	FPW2016	HM156053	Thailand NW	Tak	Phop Phra	16.38611	98.69028	21.87[b]	1401
8	UT76	HM156054	Thailand NE	Udorn Thani	Muang	17.38644	102.7883	20.86[b]	1401
9	UT169	HM156055	Thailand NE	Udorn Thani	Muang	17.38644	102.7883	21.25[b]	1401
10	UT176	HM156056	Thailand NE	Udorn Thani	Ban Phu	17.13417	102.9183	20.29[b]	1401
11	UT221	HM156057	Thailand NE	Udorn Thani	Muang	17.38644	102.7883	21.09[b]	1401
12	UT418	HM156058	Thailand NE	Udorn Thani	Muang	17.38644	102.7883	21.18[b]	1401
13	UT661	HM156059	Thailand NE	Udorn Thani	Muang	17.38644	102.7883	36.77[a]	1401
14	TM1320	HM156060	Laos	Vientiane capital	Sangthong/Samphanna	18.05579	102.31616	38.33[a]	1401
15	TM1324	HM156061	Laos	Vientiane capital	Xaysettha/Kham ngoi	17.98086	102.68386	38.95[a]	1401
16	SIDO	HM156062	Australia	NA	NA	NA	NA	26.94[a]	1401
17	Chuto	HM156063	UAE	NA	NA	NA	NA	42.82[a]	1404
r-1	TH1811	HM595489	Thailand	NA	NA	NA	NA	-ND	1401
r-2	TH1817	HM156064	Thailand	Nakorn Ratchasima	Pak Tong Chai/Pak Tong Chai	14.7167	102.0167	19.73[b]	1401
r-3	TA763	HM595490	Thailand	Ubon Ratchathani	Sirinthorn/Chong Mek	15.1333	105.4667	19.2[b]	1401
r-4	MAK243	HM595491	Taiwan	Pescadore Islands	NA	NA	NA	18.98[b]	1401
r-5-1	Kato RDD10-001	HM595493	Japan	Nigata	NA	37.9112	139.0052	21.8[b]	1401
r-5-2	Kato	L11697	Japan	Nigata	NA	37.9112	139.0052	21.44[b]	1401
r-6	Karp	L31934	New Guinea	NA	NA	NA	NA	17.56[b]	1401
r-7	Gilliam	L31933	Burma	NA	NA	NA	NA	14.9[b]	1401
r-8	Ikeda	AP008981	Japan	Nigata	NA	37.9112	139.0052	-ND	1401
r-9	Boryong	AM494475	South Korea	South Chungcheong	/ Boryong	NA	NA	-ND	1401

[a]Ct values for the10 DNA preparations obtained from blood samples of scrub typhus patients.
[b]Ct values determined for DNA preparations from cultures of patient and reference samples.
Ct, cycle threshold values; ORF, open reading frame; NA, not available; ND, not determined.

Boryong strains of *O. tsutsugamushi* (GenBank accession numbers L31934, L11697, AP008981, L31933, and AM494475, respectively) using Vector NTI advance 11 software (Invitrogen). Due to difficulties in amplifying the 47-kD HtrA gene from the *O. chuto* isolate nucleic acid, specific primers (Chur 627F and Chur 669F) were identified and selected after a partial sequence was obtained. The sequences of the primers used for PCR, nPCR, and sequencing are listed in Table 1.

The 25-μL reaction mixtures, which contained 2 μL of the nucleic acid preparations, 0.3 μM of the forward and reverse primers, and Platinum PCR SuperMix High Fidelity (Invitrogen), were incubated on a T-Gradient Thermocycler (Biometra, Goettingen, Germany) at 95°C for 2 min followed by 40 cycles of 3-step amplification at 94°C for 30 sec, 54°C for 30 sec, and 68°C for 2 min, followed by a final extension hold at 72°C for 7 min. PCR amplicons were visualized and compared to molecular weight standards (1-kbp Ladder, Invitrogen) on 1.5% agarose gels with ethidium bromide (Gibco BRL Life Technologies, Inc. Gaithersburg, MD) staining following electrophoresis. The PCR products were purified by QIAquick PCR Purification Kit (Qiagen, Valencia, CA). The sequencing reactions were performed using BigDye Terminator v 3.1 Ready Reaction Cycle Sequencing Kits (Applied Biosystems, Foster City, CA), after cleanup with Gel Filtration Cartridges (EdgeBio, Gaithersburg, MD). The reaction

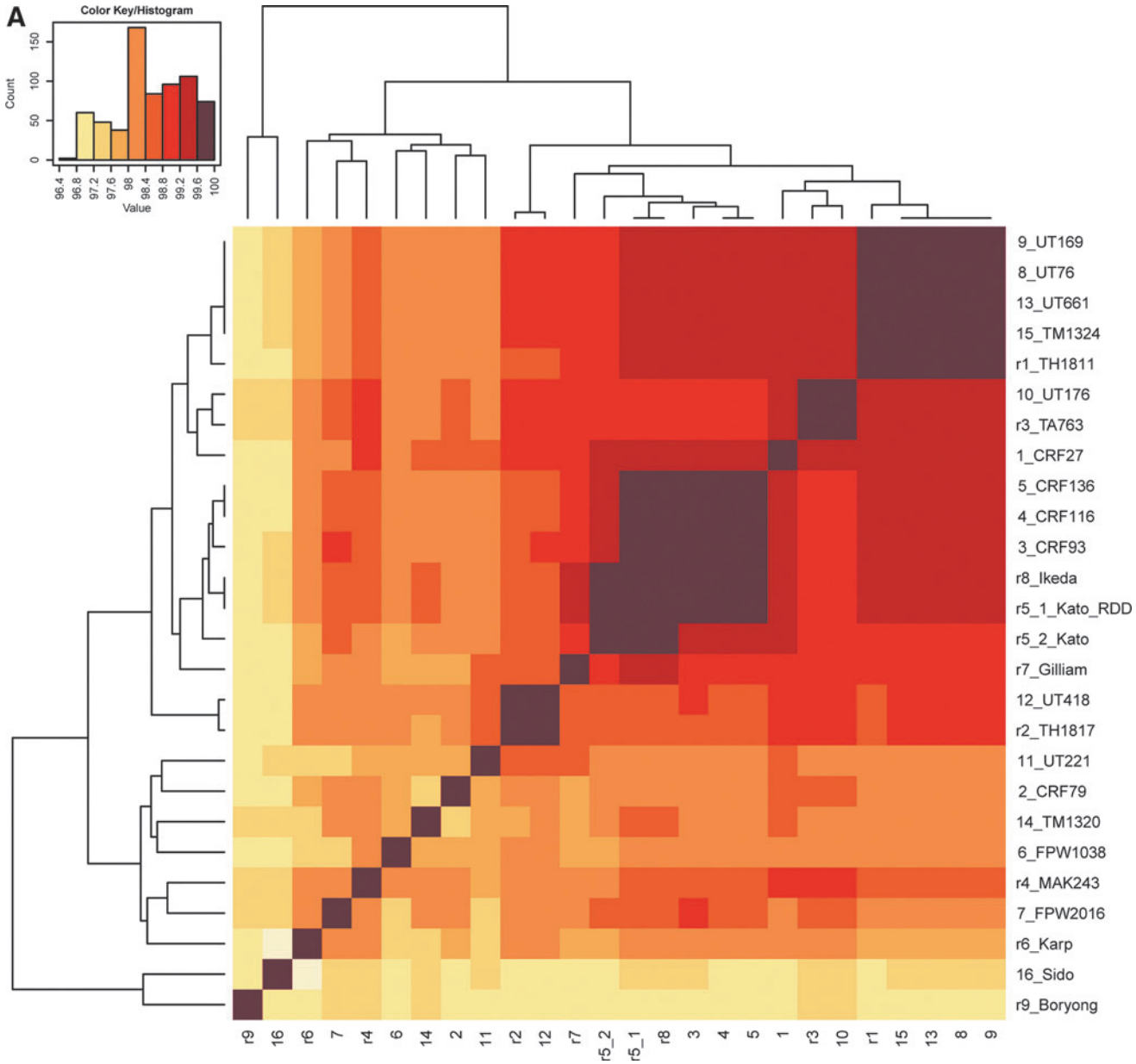

FIG. 1. Heat maps of percent identities of the 47-kD HtrA DNA (**A**) and amino acid (**B**) sequences among 25 *Orientia* isolates (without *O. chuto*). Heat maps were generated using the pairwise identity matrix tables with hierarchical clustering method. The name of each isolate was labeled on the right side of the graphs with its corresponding number code, which was used at the bottom of the graphs. Dendrograms were placed to the left side and on top of the graphs.

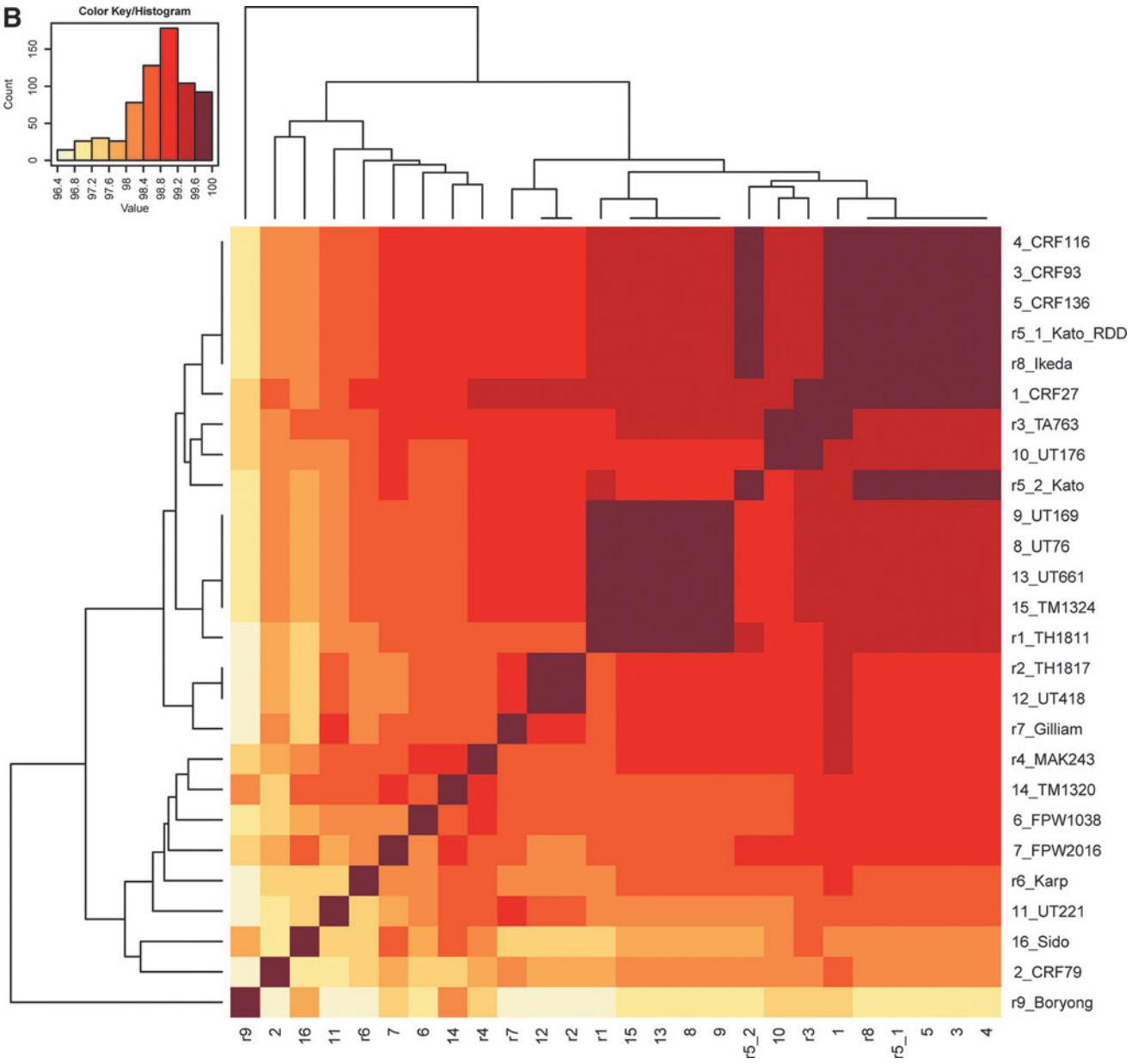

FIG. 1. (Continued).

products were run on an automated ABI Prism 3130*xl* genetic analyzer (Applied Biosystems). The sequences, from both directions of the DNA strands, were assembled by Vector NTI advance 11 software (Invitrogen), and compared to each new human isolate and with the reference strains Karp (New Guinea), Kato (Japan), Ikeda (Japan), Gilliam (Burma), and Boryong (Korea) from GenBank and Kato (RDD10-001), TA763 (Thailand), TH1811 (Thailand) TH1817 (Thailand), and MAK243 (China) sequenced in this study.

GenBank accession numbers

The 47-kD HtrA gene sequences of the new *Orientia* isolates and 5 reference strains reported herein have been deposited in GenBank with accession numbers listed in Table 2.

Phylogenetic analyses

Phylogenetic analyses were performed using MEGA5 software using the multialignment of the 47-kD HtrA gene and 47-kD protein sequences from 17 new human isolates and 9 reference strains. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura–Nei (for the 47-kD gene) and point accepted mutation (PAM) matrix-based (for 47-kD protein) models (Schwarz and Dayhoff 1979, Nei and Kumar 2000). The constructed trees were evaluated with bootstrapping from 1000 replications. The calculated translated amino acid sequences were determined by Vector NTI advance 11 software (Invitrogen). The heat maps depicting similarity/diversity of the nucleotide and amino acid sequences among 16 new human isolates

(without *O. chuto* isolate) and 9 reference strains were generated using the web tool (www.hiv.lanl.gov/content/sequence/HEATMAP/heatmap.html/).

Ethics statement

All clinical samples in this study (TM [Typhus Mahosot], CRF [Chiang Rai Fever], UT [Udorn Thani], and FPW [Fever in Pregnant Women]) were collected as part of studies investigating the causes of fever at the corresponding study sites. Ethical clearance was obtained from the Faculty of Tropical Medicine, Mahidol University (CRF, UT, FPW), the Thai Ministry of Public Health (CRF, UT, FPW), the National Ethics Committee for Health Research, Ministry of Public Health, Lao PDR (TM), and the Oxford Tropical Research Ethics Committee (all studies). All patients provided informed written consent before sample collection.

Results

qPCR Assay with DNA from patients' blood samples

All 17 dried nucleic acid preparations of *Orientia* isolates (7 from cultures and 10 from patient blood samples) sent to NMRC were positive by the Otsu47 qPCR assay. The cycle threshold (Ct) values for the 10 DNA preparations from patient blood samples represented approximately 12 to 207,157 (median = 111) genome equivalents/mL.

DNA sequence analysis

Of the 17 new isolates, CRF116 and CRF136 from northern Thailand had identical sequences, and UT76, UT169, and UT661 from northeastern Thailand and TM1324 from Laos had identical sequences within the gene studied. Thus, 13 (76.5%) new nucleotide sequences of the 47-kD HtrA gene were identified from the 17 available isolates; 10 from Thailand (4, 2, and 4 from northeastern, northwestern, and northern Thailand, respectively), and 1 each from Laos, Australia, and the UAE.

Nucleotide sequence identities of the 47-kD HtrA gene ranged from 96.7% (Karp vs. Sido) to 100% for 25 isolates without including *O. chuto* (Fig. 1a). However, the percent identities of the geographically remote *O. chuto* isolate to the other 25 isolates ranged between 84.1% and 84.6%.

The 47-kD HtrA gene sequences of 2 different passage preparations of reference strain Kato (plaque purified) archived in the NMRC, L5/90, and RDD10-001 were identical to each other and surprisingly identical to Ikeda, but

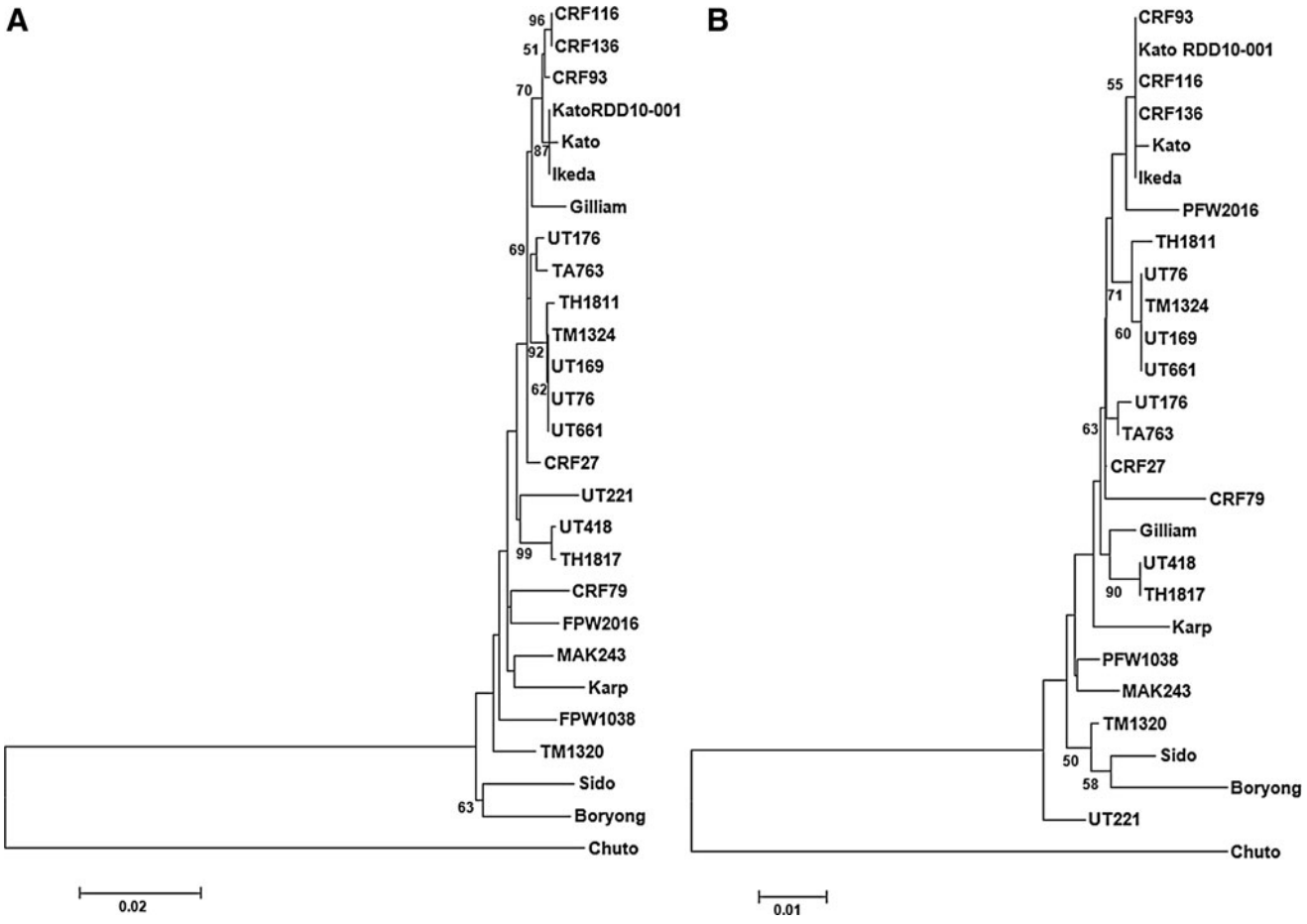

FIG. 2. Evolutionary relationships among 17 new *Orientia* isolates and 9 reference strains. Phylogenetic trees were constructed based on multiple sequence alignment of 47-kD HtrA gene open reading frame (ORF) (**A**) and calculated amino acid sequence of the 47-kD HtrA (**B**) using the maximum likelihood method. Bootstrap values above 50 are labeled at the nodes.

they had a 2-bp difference compared to the Kato sequence listed in GenBank (accession number L11697). To confirm the identity of the NMRC Kato nucleic acid preparations, 56-kD TSA sequencing was performed. Two 1114-bp sequences from the NMRC Kato preparations demonstrated 100% identity to each other and to the Kato strain 56-kD TSA gene sequence in GenBank (accession number M63382), but only 82.4% identity to the Ikeda 56-kD TSA sequence (accession number AP008981). Thus, the 2 NMRC preparations were correctly identified as belonging to the Kato strain.

Amino acid sequence analysis

The nucleotide sequences of the full ORF 47-kD HtrA gene from the 17 new isolates led to 10 new amino acid sequences. Isolates CRF93, CRF116, and CRF136 had the same translated sequence and were identical to Ikeda. Sequence of sample UT418 from northeastern Thailand was identical to the sequence obtained from TH1817. Percent identities (similarities) of calculated translated amino acid sequences for the 47-kD HtrA between the 16 new isolates (not including the *O. chuto* isolate) and 9 reference strains were 96.4% to 100% (97.4% to 100%) (Fig. 1). The identities (similarities) of the translated amino acid sequences between *O. chuto* and the other 25 isolates ranged from 82.2% to 83.3% (90.4% to 91.4%).

Phylogenetic analyses

The phylogenetic analyses conducted using the 47-kD HtrA gene among the new *Orientia* isolates and the reference strains showed that CRF93, CRF116, and CRF136 clustered together with reference strains Ikeda and Kato; UT418 joined with TH1817; UT76, UT169, UT661, and TM1324 formed a cluster with reference strain TH1811; and UT176 placed closely with TA763. The clusters formed were supported by high bootstrap values. The rest of the new isolates did not form clusters with each other or with the reference isolates (Fig. 2A). The dendrogram from the heat map by hierarchical clustering showed similar evolutionary relationships (Fig. 1A). The similar phylogenetic patterns were observed in 47-kD protein among those 26 *O. tsutsugamushi* isolates/strains (Fig. 2B).

Discussion

The aim of this study was to assess the isolate-associated divergence of the 47-kD HtrA DNA and amino acid sequences among the *Orientia* isolates studied ($n = 26$). The importance of the divergence may help explain why the vaccine candidate pKarp47 derived from a single strain of *O. tsutsugamushi*, Karp, has been found to protect mice from challenge with just 4 of 17 disparate strains of *O. tsutsugamushi* (A.L.R., unpublished data).

In agreement with previous reports that the 47-kD HtrA gene is relatively conserved (Oaks et al. 1989, Moree et al. 1992), the variation in our nucleotide sequences was seen at only 3.3% divergence among 25 isolates (not including the *O. chuto*). This compares with other conserved protein genes such as the 22-kD antigen gene, where percentage sequence divergence between 7 strains was less than 5% (Ge et al. 2005), the 58-kD GroEL protein gene with divergence under 3.5% among Korean and Thai strains (Lee et al. 2003, Paris et al. 2009), and the 16S rRNA gene with a maximum divergence of 1.5% (Ohashi et al. 1995). The conserved nature of the 47-kD HtrA nucleotide sequence stands in stark contrast to that of the 56-kD TSA gene, where divergence among isolates of up to and greater than 80% were noted (Blacksell et al. 2008, Fournier et al. 2008, Kelly et al. 2009). Moreover, the 9 distinct clusters of genotypes described for the 56-kD TSA gene were not seen with the phylogenetic tree constructed for the 47-kD HtrA gene (Fig. 1A). However, even though the 47-kD HtrA gene is relatively conserved within the genus, *Orientia* isolates could be seen to cluster based upon differences in nucleotide sequences. The 4 clusters identified included: (1) UT661, TM1324, UT76, UT169, and TH1811; (2) Kato, Ikeda, CRF93, CRF116, and CRF136; (3) UT418 and TH1817; and (4) TA763 and UT176. In addition, certain *Orientia* isolates did not cluster similarly in both the 47-kD HtrA and 56-kD TSA phylogenetic trees (*e.g.*, Kato and Ikeda).

Further characterization of the 47-kD HtrA protein among *Orientia* isolates included calculating the amino acid sequences; as with the nucleotide sequences, unique differences were seen between *Orientia* isolates (Fig. 1B). The greatest difference was seen between the amino acid sequence of the *O. chuto* isolate from the UAE and all other isolates, including reference strains, supporting the evidence that this isolate represents a new species of *Orientia*, as recently reported (Izzard et al. 2010). The large divergence was seen more impressively when unique differences were identified in the nucleotide sequences of the 56-kD TSA ($n = 31$) and 16S rRNA ($n = 9$) gene of the *O. chuto* when compared to *O. tsutsugamushi* (Izzard et al. 2010).

Additional differences in the amino acid sequences from the consensus sequence were seen in the Boryong, Sido, and Karp isolates. The observation made that the Karp strain was divergent from other isolates based upon the 47-kD HtrA sequence was unexpected because it has been considered the type strain of *O. tsutsugamushi*. This divergence is also notable in the 56-kD phylogenetic analyses, which showed Karp and similar isolates grouping away from other strains and isolates (Kelly et al. 2009). In addition, a phylogenetic analysis of 22-kD protein gene sequences of 7 *O. tsutsugamushi* isolates also suggested that the Karp strain is relatively distant from the other isolates (Ge et al. 2005). This implies that vaccine candidates based upon the Karp strain may need to be augmented by other gene/gene products from isolates more represented in the genus and especially from those isolates associated with human disease, *i.e.*, obtained from scrub typhus patients (Ruang-Areerate et al. 2011).

In contrast to the overall large extent of amino acid conservation displayed among 25 *Orientia* isolates, the bulk of the observed amino acid substitutions were associated with *O. chuto*. The observed amino acid sequence changes could suggest that the 47-kD HtrA protein may be able to withstand a large amount of variation, at least in the UAE, where the ecology maybe significantly different from previously described endemic regions of Asia, northern Australia, and the western Pacific. A less plausible alternative is that although the protein is expressed, it is nonfunctional. The ability of *O. chuto* to survive/thrive needs further investigation.

Acknowledgments

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Author Disclosure Statement

There is no commercial relationship between any of the authors and products used in the study, thus no competing financial interests exist.

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