

## Isolation of a Novel *Orientia* Species (*O. chuto* sp. nov.) from a Patient Infected in Dubai<sup>∇</sup>

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Received 29 July 2010/Returned for modification 8 September 2010/Accepted 17 September 2010

**In July 2006, an Australian tourist returning from Dubai, in the United Arab Emirates (UAE), developed acute scrub typhus. Her signs and symptoms included fever, myalgia, headache, rash, and eschar. *Orientia tsutsugamushi* serology demonstrated a 4-fold rise in antibody titers in paired serum collections (1:512 to 1:8,192), with the sera reacting strongest against the Gilliam strain antigen. An *Orientia* species was isolated by the *in vitro* culture of the patient's acute blood taken prior to antibiotic treatment. The gene sequencing of the 16S rRNA gene (*rrs*), partial 56-kDa gene, and the full open reading frame 47-kDa gene was performed, and comparisons of this new *Orientia* sp. isolate to previously characterized strains demonstrated significant sequence diversity. The closest homology to the *rrs* sequence of the new *Orientia* sp. isolate was with three strains of *O. tsutsugamushi* (Ikeda, Kato, and Karp), with a nucleotide sequence similarity of 98.5%. The closest homology to the 47-kDa gene sequence was with *O. tsutsugamushi* strain Gilliam, with a nucleotide similarity of 82.3%, while the closest homology to the 56-kDa gene sequence was with *O. tsutsugamushi* strain TA686, with a nucleotide similarity of 53.1%. The molecular divergence and geographically unique origin lead us to believe that this organism should be considered a novel species. Therefore, we have proposed the name "*Orientia chuto*," and the prototype strain of this species is strain Dubai, named after the location in which the patient was infected.**

The causative agent of scrub typhus, the obligate intracellular bacterium *Orientia tsutsugamushi*, is transmitted by the bite of infected chigger mites (primarily *Leptotrombidium* spp.) to humans. It is known to occur within a larger-than 8-million-km<sup>2</sup> region, from Siberia in the north, the Kamchatka Peninsula in the east, Pakistan in the west, and down to Australia in the south (Queensland, the Northern Territory, Western Australia, and parts of Oceania) (1, 5, 15). It is estimated that more than one million cases of scrub typhus occur each year (16), although accurate incidence rates are hard to obtain as infection often occurs in rural areas where diagnostic facilities are limited.

In 1995, after the extensive reanalysis of the genus *Rickettsia*, the organism *Rickettsia tsutsugamushi* was reclassified under the new genus *Orientia* and was renamed *Orientia tsutsugamushi* gen. nov. (12). Although this is the only species in the genus, there are numerous genotypically and antigenically disparate isolates/strains. In the past, new isolates of *O. tsutsug-*

*amushi* were compared only to Kato, Karp, and Gilliam strains (2). With advances in diagnostic methods, the number of different serotypes has increased dramatically (8). However, all of the previous strains were genetically very similar (9).

This study focuses on a divergent isolate of *Orientia* sp. ("*Orientia chuto*") that originated in Dubai, United Arab Emirates (UAE), which is more than 500 km west of the known scrub typhus region. While *O. tsutsugamushi* has been found in temperate zones and even semiarid climates, high levels of infection occur primarily in the tropics, usually in areas of dense scrub (14). This isolate, however, was obtained from an Australian resident who had traveled only in the UAE and United Kingdom. The isolate was obtained when the patient was admitted to the Alfred Hospital, Victoria, Australia, and was grown *in vitro* at the Australian Rickettsial Reference Laboratory, Victoria, Australia, where it was analyzed to determine its degree of genetic divergence. A new real-time PCR assay was developed to detect this novel isolate.

A 52-year-old female presented to her local general practitioner (GP) in mid-July 2006. She had traveled to Dubai and the United Kingdom prior to the onset of her illness. During her stay in Dubai, she visited a stable where there were horses,

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<sup>∇</sup> Published ahead of print on 6 October 2010.

## Report Documentation Page

*Form Approved*  
*OMB No. 0704-0188*

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1. REPORT DATE <b>SEP 2010</b>	2. REPORT TYPE	3. DATES COVERED <b>00-00-2010 to 00-00-2010</b>			
4. TITLE AND SUBTITLE <b>Isolation of a Novel Orientia Species ( <i>O. chuto</i> sp. nov.) from a Patient Infected in Dubai</b>		5a. CONTRACT NUMBER			
		5b. GRANT NUMBER			
		5c. PROGRAM ELEMENT NUMBER			
6. AUTHOR(S)		5d. PROJECT NUMBER			
		5e. TASK NUMBER			
		5f. WORK UNIT NUMBER			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) <b>Naval Medical Research Center, Viral and Rickettsial Diseases Department, Silver Spring, MD, 20910</b>		8. PERFORMING ORGANIZATION REPORT NUMBER			
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)		10. SPONSOR/MONITOR'S ACRONYM(S)			
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)			
12. DISTRIBUTION/AVAILABILITY STATEMENT <b>Approved for public release; distribution unlimited</b>					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT <b>unclassified</b>	b. ABSTRACT <b>unclassified</b>	c. THIS PAGE <b>unclassified</b>	<b>Same as Report (SAR)</b>	<b>5</b>	

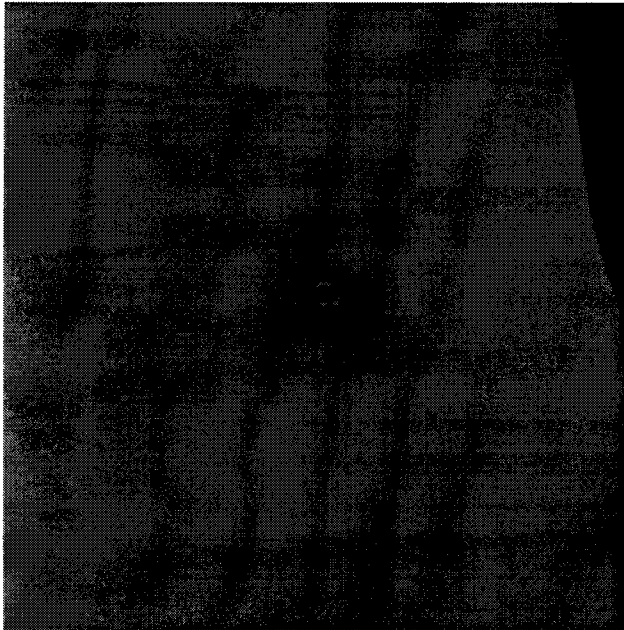


FIG. 1. Eschar on the abdomen of the patient.

dogs, and cats (day 0). She noticed an eschar on her abdomen 11 days later while on the United Kingdom leg of her journey (Fig. 1). Upon returning to Australia on day 16, she developed lymphadenopathy and complained of general myalgia (day 17), followed by fever and rash (day 18). On day 21 she developed a maculopapular rash over her abdomen and complained of headache, pain behind her eyes, generalized myalgia, and backache, and initially she was diagnosed with a viral illness by her GP. A full blood examination taken on this day showed unremarkable results, with hemoglobin levels of 137 g/liter (normal range, 115 to 165 g/liter), white cell count of  $7.8 \times 10^9$ /liter (normal range,  $4 \times 10^9$  to  $11 \times 10^9$ /liter), platelet levels of  $185 \times 10^9$ /liter (normal range,  $150 \times 10^9$  to  $400 \times 10^9$ /liter), an erythrocyte sedimentation rate (ESR) of 12 mm/h (normal is <20 mm/h), and elevated C-reactive proteins (CRP) at 21.3 mg/liter (normal, <5 mg/liter). Liver function was abnormal, with alanine transaminase (ALT) levels of 57 U/liter (normal, <35 U/liter) and aspartate aminotransferase (AST) levels of 57 U/liter (normal, <40 U/liter).

Three days later (day 24) she was seen by an infectious diseases physician, who found that she was afebrile with faint macules on her abdomen and an eschar on her upper abdomen. She had mild hepatomegaly but no splenomegaly. Blood tests for a number of pathogens were performed and showed a positive total antibody titer of 1:512 for scrub typhus group (STG) rickettsia by indirect fluorescence antibody assay (IFA) (Fig. 2). *Orientia* sp. organisms later were isolated *in vitro* from a blood sample collected on day 24 postexposure. The physician diagnosed her with a rickettsial infection and started doxycycline treatment (100 mg twice a day) for 14 days. Two days after treatment commenced (day 26) the patient was admitted to hospital due to ongoing fevers and lethargy.

A full blood examination on admission showed the white cell count had risen to  $13.4 \times 10^9$ /liter, and her lymphocytes had

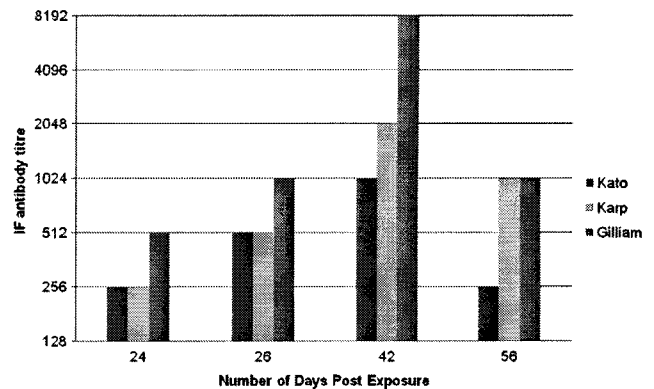


FIG. 2. Scrub typhus serology showing a marked change in antibody titers when tested against three strains of *O. tsutsugamushi* during a 56-day period.

risen to  $8.3 \times 10^9$ /liter (normal range,  $1.0 \times 10^9$  to  $4.0 \times 10^9$ /liter). Her monocytes also were elevated, at  $2.1 \times 10^9$ /liter (normal range,  $0.1 \times 10^9$  to  $1.0 \times 10^9$ /liter). A blood film also showed the presence of numerous atypical (reactive) lymphocytes. Her ESR was 14 mm/h and the CRP was 39 mg/liter. Liver function tests revealed that her ALT has risen to 181 U/liter, AST to 176 U/liter, and alkaline phosphatase (ALP) to 150 U/liter (ALP normal range, 25 to 100U/liter). Her gamma-glutamyl transpeptidase ( $\gamma$ GT) level also had risen to 109 U/liter (normal range, <30 U/liter).

After the completion of the 14-day course of doxycycline her liver function had normalized. By day 34, the patient's fever and sweats had resolved and myalgia and rash were noticeably reduced. By day 42, the patient reported feeling markedly better, although still quite lethargic. STG rickettsia serology titer peaked at 1:8,192. Day 56 STG rickettsia serology demonstrated a decrease in titer to 1:1,024 (Fig. 2). By day 90 the patient still was reporting lethargy.

#### MATERIALS AND METHODS

**IFA.** Serum taken from the patient was tested for the presence of STG antibodies using a microimmunofluorescence assay (IFA). The sample first was tested at 1:128 in 2% casein-phosphate buffered saline (PBS). It then was incubated for 30 min at 37°C on a slide containing separate wells of Kato, Karp, and Gilliam antigens. The slide then was rinsed in PBS before a secondary anti-human total immunoglobulin antibody with a fluorescein isothiocyanate (FITC) label (Kirkegaard & Perry Laboratories) was added. The slide was incubated for a second time at 37°C for 30 min and then washed again. The slide then was viewed using a Leica DM LS microscope (Leica, Germany) with an ultraviolet epifluorescence illuminator. The positive sample then was titrated to the end point (4).

**Culture.** An EDTA blood tube was collected (along with the first serum sample) prior to doxycycline treatment. This was spun at  $6,000 \times g$  for 5 min to separate the buffy coat from the red blood cells and plasma. The buffy coat was used to inoculate a Vero cell line, which was incubated for 2 weeks before screening with antisera for *O. tsutsugamushi* by direct IF.

**DNA extraction and PCR assays.** DNA was extracted from the buffy coat and cell culture isolate using the Real Genomics gene DNA extraction kit (Real Biotechnology Corporation, Taiwan) per the manufacturer's methods, and PCR assays were performed on the extracted DNA (see below).

**16S rRNA (*rrs*) gene.** The isolate DNA underwent *rfs* gene amplification by conventional PCR assay using primers previously described (3).

**56-kDa gene.** The 56-kDa gene assay was used for obtaining the partial sequences of the highly variable 56-kDa outer membrane protein gene of the cell culture isolate. The method involved a previously described nested conventional PCR, with the reaction end volume reduced from 100 to 25  $\mu$ l (6).

TABLE 1. qPCR primer and probe set sequences targeting the *rrs* gene

Name	Sequence (5' to 3')
CH-F	.....GGA GGA AAG ATT TAT CGC TGA TGG
CH-R	.....TAG GAG TCT GGG CCG TAT CTC
CH-P	.....FAM d(TGT GGC TGT CCG TCC TCT CAG ACC) BHQ-1 <sup>a</sup>

<sup>a</sup> FAM, 6-carboxyfluorescein; BHQ-1, black hole quencher 1.

Four primers (2 primer sets) were used, leading to a resulting amplicon of approximately 620 bp. The nucleotide sequences of primers used were as follows: outer primer pair RTS-8 (5'-AGGATTAGAGTGTGGTCCTT-3') and RTS-9 (5'-ACAGATGCACTATTAGGCAA-3'), followed by inner primer pair RTS-6 (5'-GTTGGAGGAATGATTACTGG-3') and RTS-7 (5'-AGCGCTA GGTT ATTAGCAT-3'). The first-step PCR amplification used primers RTS-8 and RTS-9, and the second step used primers RTS-6 and RTS-7. One microliter of DNA extract was used as the template in the first step of nested PCR, and 0.5  $\mu$ l of the primary PCR product was used as the template for the second amplification. All reactions were performed in a volume of 25  $\mu$ l, containing DNA template, 2.5  $\mu$ l of 10 $\times$  PCR buffer (Promega Co.), 2.5 U of *Taq* DNA polymerase (Promega), and 200  $\mu$ M (each) deoxynucleoside triphosphates (dNTPs). MgCl<sub>2</sub> at end concentrations of 4 and 2 mM was added to the first and second steps of the nested PCR, respectively. The amplification conditions were heat denaturation at 94°C for 1 min, annealing at 55°C for 1.5 min, and polymerization at 72°C for 2 min in the first step of the nested PCR. In the second step, one cycle consisted of heat denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and polymerization at 72°C for 1 min. Amplification proceeded for 30 cycles each in the first and second step of the nested PCR using a DNA thermal cycler (PTC-200, MJ Research). All PCR products were stained with ethidium bromide and visualized by agarose gel electrophoresis.

**47-kDa gene.** Standard PCR and nested PCR (nPCR) were performed to amplify the entire open reading frame (ORF) of the 47-kDa HtrA gene of the cell culture isolate. Primers used were selected from conserved regions of the 47-kDa HtrA gene for PCR (Ot-145F and Ot-1780R) and nPCR (Ot-263F and Ot-1133R) (J. Jiang, S. Blacksell, D. Paris, N. Aukkanit, P. Newton, R. Phetsouvanh, L. Izzard, J. Stenos, S. Graves, X. Hu, A. Wallqvist, N. Day, and A. Richards, unpublished data), which successfully amplified a segment of the 47-kDa HtrA gene. Due to difficulties in amplifying the ORF of the 47-kDa HtrA gene using other conserved primers, specific primers (Chur 627F and Chur 669F) were identified and selected from the partial sequence obtained, and PCR and nPCR were performed again using the specific primers with reverse primer Ot-1404R. The 25- $\mu$ l reaction mixtures, which contained Platinum PCR Super-Mix High Fidelity (Invitrogen), 2  $\mu$ l of the nucleic acid preparations, and 0.3  $\mu$ M the forward and reverse primers, were incubated at 95°C for 2 min, followed by 40 cycles of three-step amplification at 94°C for 30 s, 54°C for 30 s, and 68°C for 2 min, followed by a final extension hold at 72°C for 7 min on a T-Gradient thermocycler (Biotetra, Goettingen, Germany). PCR amplicons were visualized

on 1.5% agarose gels with ethidium bromide (Gibco BRL Life Technologies, Inc. Gaithersburg, MD) staining following electrophoresis.

**Sequencing.** The resulting amplicons of the *rrs* and 56-kDa assays underwent commercial DNA sequencing (Macrogen, South Korea) using BigDye terminator cycling conditions on an automated nucleotide sequencer (model ABI3730XL; Applied Biosystems). These sequences then underwent BLAST analysis for the confirmation and characterization of the infecting microorganism.

The amplicons of the 47-kDa gene were purified by the QIAquick PCR purification kit (Qiagen, Valencia, CA) before being sequenced. The sequencing reactions were performed using BigDye terminator v 3.1 ready reaction cycle sequencing kits (Applied Biosystems, Foster City, CA) and run on an automated ABI Prism 3130xl genetic analyzer (Applied Biosystems). The sequences, from both directions of the DNA strands were assembled by Vector NTI advance 11 software (Invitrogen).

**Design of a quantitative real-time PCR assay.** The *rrs* gene sequence of *O. chuto* and all available *O. tsutsugamushi* *rrs* gene sequences in NCBI (accessed on 20 May 2008) were aligned using the Clustal W algorithm within the MEGA-4 software package (13). Based on the resulting consensus sequence, a new TaqMan primer set and probe targeting the *rrs* gene were designed using the Alle-ID 3 software package (Table 1). The assay was tested against the DNA from other medically important bacteria mentioned previously (11) and other rickettsiae to test its specificity. The assay's target region was cloned using the TA cloning kit (Invitrogen) per the manufacturer's instructions. The plasmid then was extracted using a QuickLyse Mini Prep kit (Qiagen, Germany). The plasmid concentration was determined using an ND-1000 spectrophotometer (NanoDrop Technologies), from which the number of copies of the extracted plasmid per microliter was calculated. Using 10-fold dilutions, the extracted plasmid was titrated from an initial concentration of  $9.9 \times 10^{10}$  copies down to 9.9 copies, and each dilution was analyzed using the new quantitative PCR (qPCR) assay. By comparing the number of copies at this point to that of the endpoint dilution, the sensitivity of the assay was determined.

## RESULTS

**Serology.** Serum samples were collected from the patient on days 24, 26, 42, and 56 postinfection. On day 24, the serological results showed a titer of 1:512 against the Gilliam strain and 1:256 against the Kato and Karp serotypes. The titers peaked by day 42, with antibodies against the Gilliam strain showing a 5-fold increase in titer (1:8,192), while during the same time antibody levels against Kato and Karp strains demonstrated a 2-fold (1:1,024) and 3-fold (1:2,048) increase in titers, respectively. By day 56 the titers had decreased to 1:256 for Kato and 1:1,024 for both Karp and Gilliam strains, respectively (Fig. 2).

TABLE 2. Percentage pairwise divergence plot of the *rrs* gene of *O. chuto* strain Dubai with various strains of *O. tsutsugamushi* showing the significant level of divergence of *O. chuto* strain Dubai

Species and strain	% sequence divergence from:									
	<i>O. chuto</i>	<i>O. tsutsugamushi</i> strain								
		Ikeda	Boryong	Kato	Karp	Gilliam	Kuroki	Kawasaki	Shimokoshi	Litchfield
<i>O. chuto</i>										
<i>O. tsutsugamushi</i>										
Ikeda	1.5									
Boryong	1.6	0.3								
Kato	1.5	0.0	0.3							
Karp	1.5	0.2	0.1	0.2						
Gilliam	1.7	0.5	0.2	0.5	0.3					
Kuroki	1.6	0.3	0.0	0.3	0.1	0.2				
Kawasaki	1.9	0.4	0.5	0.4	0.4	0.6	0.5			
Shimokoshi	2.0	1.0	1.1	1.0	1.0	1.1	1.1	0.9		
Litchfield	1.9	0.6	0.4	0.6	0.4	0.5	0.4	0.6	1.1	

TABLE 3. Percentage pairwise divergence plot of the 47-kDa gene of *O. chuto* strain Dubai with from those of strains of *O. tsutsugamushi* showing the significant level of divergence from *O. chuto* strain Dubai

Species and strain	% sequence divergence from:						
	<i>O. chuto</i>	<i>O. tsutsugamushi</i> strain					
		Gm47	Ikeda	Br47	Kp47	Pkt5	Boryong
<i>O. chuto</i>							
<i>O. tsutsugamushi</i>							
Gm47	17.7						
Ikeda	17.8	0.6					
Br47	17.9	3.3	3.1				
Kp47	18.0	2.3	2.0	3.2			
Pkt5	18.0	0.8	0.1	3.2	2.1		
Boryong	18.2	3.1	2.8	0.8	3.2	3.0	

**Culture.** Vero cell monolayers were inoculated with 100 µl fresh buffy coat from the patient prior to the initiation of antibiotic treatment (day 24) under sterile conditions. Tissue culture flasks were centrifuged at 500 × g for 30 min and then incubated at 35°C (5% CO<sub>2</sub>). No medium changes were performed, and after 2 weeks a direct IF assay was performed to test for the presence of *Orientia* sp. within Vero cells.

**Molecular analysis.** Pairwise analysis of this isolate with various strains of *O. tsutsugamushi* showed that the new *Orientia* sp. isolate had the highest level of divergence within the 16S rRNA (*rrs*), 47-kDa, and 56-kDa genes (Tables 2 and 3). The analysis of the *rrs* sequence showed the closest phylogenetic relative as *O. tsutsugamushi* strains Ikeda, Kato, and Karp, with sequence similarities of 98.5%. The analysis of the 47-kDa gene and all sequences that were available in the NCBI database showed the closest phylogenetic relative to be *O. tsutsugamushi* strain Gilliam, with a percent similarity of 82.3% (Table 2). The analysis of a 614-bp fragment of the 56-kDa gene and the strains of *O. tsutsugamushi* mentioned previously (2) showed the closest phylogenetic relative to be *O. tsutsugamushi* strain TA686, with a percent similarity of 53.1% (data not shown).

The phylogenetic relationships between each of the gene sequences of *O. chuto* strain Dubai and various strains of *O. tsutsugamushi* were analyzed using the algorithms mentioned above. The 16S rRNA (*rrs*), 56-kDa, and 47-kDa gene sequence analyses using the neighbor-joining algorithm are shown in Fig. 3 to 5.

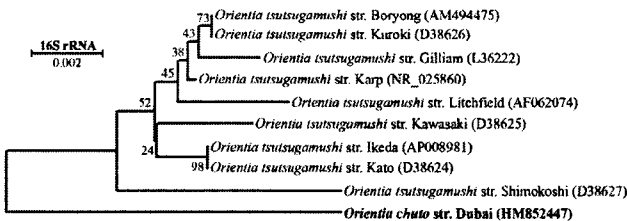


FIG. 3. Phylogenetic tree showing the 16S rRNA gene of *Orientia chuto* strain Dubai among *O. tsutsugamushi* strains. The tree was prepared using the neighbor-joining algorithm within the Mega 4 software. Bootstrap values are indicated at each node. The scale bar represents a 0.2% nucleotide divergence.

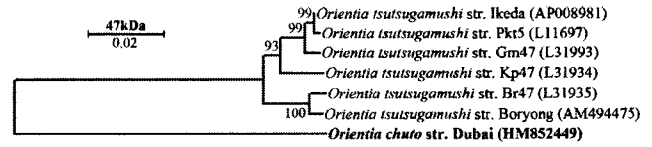


FIG. 4. Phylogenetic tree showing the 47-kDa gene of *Orientia chuto* strain Dubai among *O. tsutsugamushi* strains. The tree was prepared using the neighbor-joining algorithm within the Mega 4 software. Bootstrap values are indicated at each node. The scale bar represents a 2.0% nucleotide divergence.

**qPCR.** A real-time qPCR (targeting *rrs*) performed on the new *Orientia* sp. isolate culture was positive, and a 141-bp amplicon was observed when the product was run on an agarose gel. The sensitivity of the assay, determined by performing real-time PCRs on titrated plasmid samples ranging from  $9.9 \times 10^{10}$  to 9.9 copies in size, was shown to be 9.9 template copies per reaction. The threshold cycle ( $C_T$ ) values ranged from 3.52 ( $9.9 \times 10^{10}$  copies) to 35.86 ( $9.9 \times 10^0$  copies).

When tested with *O. tsutsugamushi* strains Kato, Karp, and Gilliam, the same assay produced a positive result for all three strains, and a band around the 141-bp mark was observed when the products were run on an agarose gel. The assay produced negative results when tested against the DNA of other medically important bacteria previously used (11), and no band was evident around the 141-bp mark when run on an agarose gel.

DISCUSSION

In this study, the patient contracted scrub typhus in Dubai within the UAE, which is well outside the recognized so-called tsutsugamushi triangle (Fig. 6). Typically scrub typhus is considered a tropical disease, with the majority of cases occurring in rural Asian tropics, although cases have been reported in

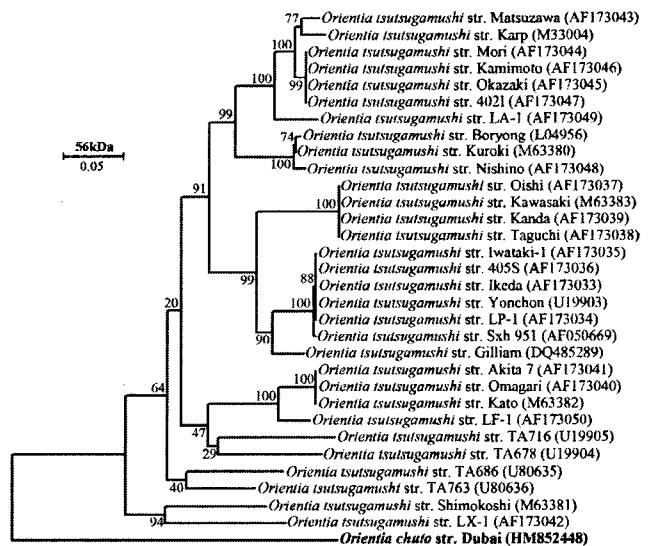


FIG. 5. Phylogenetic tree showing the 56-kDa gene of *Orientia chuto* strain Dubai among *O. tsutsugamushi* strains. The tree was prepared using the neighbor-joining algorithm within the Mega 4 software. Bootstrap values are indicated at each node. The scale bar represents a 5.0% nucleotide divergence.

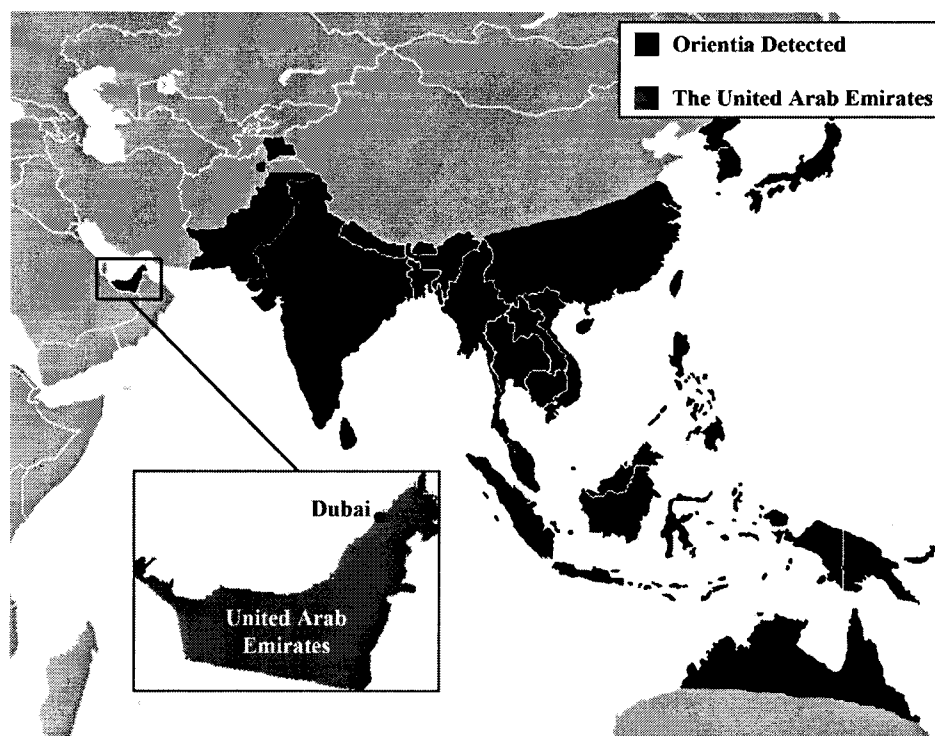


FIG. 6. Regional map showing the distribution of scrub typhus and the location of Dubai within the UAE. (Modified from a map by Lokal Profil that is freely available on Wikipedia.)

temperate zones (15). The habitats within which transmission usually occurs are areas where scrubland has been allowed to grow after deforestation (15). The natural environment of the UAE is desert with scant vegetation. More recently, however, the environment has been altered considerably, with millions of trees having been planted. Even so, this would not be considered an ideal environment for mites and *O. tsutsugamushi*.

Samples collected from the patient were positive for the presence of scrub typhus group antibodies using IFA serology, showing a clear rise in titer. The testing of the patient's acute blood sample for the presence of *Orientia* sp. DNA was positive using a specially designed new qPCR assay that was based on the *rrs* sequence. The Vero culture inoculated with the same preantibiotic buffy coat sample as that used for the qPCR assay was examined after 2 weeks of growth using direct IF and revealed the presence of a scrub typhus group rickettsiae growing within the cytoplasm of the VERO cells. qPCR analysis of the culture using the new *rrs* assay also was positive. This new qPCR assay has the potential to be used in a clinical setting for the diagnosis of all *Orientia* species, as it detected all isolates tested.

The conventional amplification of the *rrs* and 56-kDa genes was unsuccessful using previously described assays routinely used within our laboratory (7, 10). With the use of a different set of *rrs* gene primers, a multiple mixture of various 56-kDa primers, and custom-designed 47-kDa primers, we were able to successfully amplify sections of all three genes. The amplified genes showed a level of diversity noticeably greater than that of any previously identified strain of *O. tsutsugamushi*, which helps to explain the difficulties with the initial amplification.

For example, it is noted that the majority of *O. tsutsugamushi* strains have less than a 1% divergence within their *rrs* gene sequences, and that *O. tsutsugamushi* strain Shimokoshi was considered the most divergent, as it had a percent divergence of around 1.5%. In contrast, this new isolate has a percent divergence with its *rrs* gene of 2% and sits well outside the normal *O. tsutsugamushi* cluster (Fig. 3). *O. tsutsugamushi* strain Shimokoshi also was considered the most divergent strain in 56-kDa gene analyses, with a divergence of 31 to 37% (2). However, *O. chuto* strain Dubai had a divergence of 47 to 58%, which was much greater than that of *O. tsutsugamushi* strain Shimokoshi. The 47-kDa gene of *O. chuto* strain Dubai also was significantly divergent from those of various strains of *O. tsutsugamushi*. Among the seven *O. tsutsugamushi* 47-kDa sequences compared, the level of divergence was between 0.1 and 3.3%; however, the 47-kDa gene of *O. chuto* strain Dubai had a divergence of 17.7 to 18.2%.

Although no criteria have been established to define a new *Orientia* species, the unique molecular sequences combined with the geographically unique origin of this isolate lead us to claim that it constitutes a new *Orientia* species. Consequently, we propose the new species name *Orientia chuto*, with "chuto" being Japanese for "Middle East," with the prototype strain of this species being strain Dubai, named after the location in which the patient was infected.

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