A highly sensitive quantitative real-time PCR assay based on the groEL gene of contemporary Thai strains of Orientia tsutsugamushi

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Abstract

Partial nucleotide sequences (459 bp) of the groEL gene (encoding the 60-kDa heat shock protein, HSP60) from 23 contemporary isolates of Orientia tsutsugamushi isolated from patients with acute scrub typhus in Thailand were compared with 16 reference strain sequences to evaluate the potential of groEL as a conserved and representative target for molecular diagnostics.. Overall nucleotide identity within all available O. tsutsugamushi isolates (n = 39) was 98.8% (range: 95.0–100), reflecting a high degree of conservation; nucleotide identities were 67.5% and 65.6%, respectively, when typhus and spotted fever group rickettsiae were included.. A highly sensitive and quantitative real-time PCR assay was designed and evaluated using 61 samples, including buffy coats from patients in Thailand and Laos. Reliable and accurate quantitation of bacterial loads allows further investigation of other diagnostic methods and may lead to an improved understanding of the pathophysiology of acute scrub typhus, an important but under-recognized disease.

Keywords: Diagnostics, groEL, heat shock protein, phylogenetics, real-time PCR, scrub typhus

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Introduction

The Rickettsiaceae family consists of a group of highly fastidious, obligate intracellular Gram-negative organisms. They are divided into three groups, based on antigenic reactivity—the scrub typhus group, typhus group (TG) and spotted fever group (SFG). Scrub typhus, caused by Orientia tsutsugamushi, and murine typhus, caused by Rickettsia typhi, are the most common forms of Typhus in rural Thailand and Laos, accounting for 20–30% of undifferentiated fevers [1–4].

Problems in under-recognition of rickettsial illnesses, mainly due to diagnostic difficulties, lead to delay and errors in patient management. The development of rapid, inexpensive and accurate diagnostic methods is necessary, both to improve diagnosis and to promote awareness of these potentially serious but treatable diseases in highly populous rural areas of Southeast Asia.

With the increasing availability of gene sequences, allowing the exploitation of more gene-based targets, molecular assays have been developed and evaluated for the diagnosis of acute scrub typhus. A common target gene used in nested conventional as well as real-time PCR assays, encodes the 56-kDa outer membrane protein [5,6]. Another target gene encodes the 47-kDa outer membrane protein, used in a real-time PCR assay [7].

Quoted sensitivities for the nested 56-kDa assays range from 62% to 90%, with specificities approaching 100% when compared to the reference standard immunofluorescence assay (IFA) [5,6,8,9]. Recent studies have characterized the 60-kDa heat shock protein GroEL of x-proteobacteriaceae as a molecular indicator of various forms of cellular stress. GroEL production is upregulated during the early period of infection, leading to high-level expression of essential proteins in eubacterial genomes and in eukaryotic organelles [10,11]. The most prominent protein of Rickettsia conorii (SFG), revealed by two-dimensional PAGE proteomic analysis and reacting with antibodies in rabbit and patient sera, was a 60-kDa protein identified as GroEL [12].

The corresponding gene has been proposed as a target for molecular diagnostics for differentiation between members of the genus Rickettsia [13] and the family Anaplasmato-
ceae [14]. Recently, Park et al. described the use of a conventional duplex PCR assay, based on the groEL gene, for the detection of rickettsiae and the identification of *O. tsutsugamushi* [15]. This assay was evaluated using a limited number of *O. tsutsugamushi* reference type strains, including strains Karp, Kato, Kawasaki, Gilliam and Boryong.

The groEL nucleotide sequences of 23 contemporary *in vitro* isolates of *O. tsutsugamushi* isolated from patients with scrub typhus in Thailand were determined and the corresponding isolates cultured in this study (Table 1) were collected from scrub typhus patients (5 ml full blood containing EDTA) at two sites in Thailand during 2004–2005. Previous antibiotic use was an exclusion criterion for sample collection. The isolates were cultivated in VERO cell monolayers in 25-cm² polystyrene tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ, USA) containing RPMI-1640 medium supplemented with L-glutamine, HEPES (2 mM) and fetal bovine serum (FCS Gold; PAA, Laboratories GmbH, Pasching, Austria) (10%, v/v)). Cultures of *O. tsutsugamushi* were incubated at 35°C in a 5% CO₂ atmosphere [16]. When the cytopathic plaque-formation reached 90–100% confluency of the whole monolayer, the cells were harvested, pelleted and stored at −80°C.

The buffy coat samples (from 5 ml of full blood containing EDTA) for real-time PCR were collected from patients with scrub typhus, who gave informed consent, at Udon Thani Hospital, north-eastern Thailand, and at Mahosot Hospital, Vientiane, Laos PDR.

The present study was approved by the Ministry of Public Health, Royal Government of Thailand (Thailand), the Faculty of Medical Sciences Ethical Review Committee, the National University of Laos (PDR Laos) and the Oxford Tropical Research Ethics Committee (OXTREC, UK). The *O. tsutsugamushi* isolates cultured in this study (Table 1) were collected from scrub typhus patients (5 ml full blood in EDTA), which gave informed consent, at two sites in Thailand during 2004-2005.

### Materials and Methods

#### Clinical samples

The *O. tsutsugamushi* isolates cultured in this study (Table 1) were collected from scrub typhus patients (5 ml of full blood containing EDTA) at two sites in Thailand during 2004–2005. Previous antibiotic use was an exclusion criterion for sample collection. The isolates were cultivated in VERO cell monolayers in 25-cm² polystyrene tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ, USA) containing RPMI-1640 medium supplemented with L-glutamine, HEPES (2 mM) and fetal bovine serum (FCS Gold; PAA, Laboratories GmbH, Pasching, Austria) (10%, v/v)). Cultures of *O. tsutsugamushi* were incubated at 35°C in a 5% CO₂ atmosphere [16]. When the cytopathic plaque-formation reached 90–100% confluency of the whole monolayer, the cells were harvested, pelleted and stored at −80°C.

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### TABLE 1. Details of isolates and strains used in this study

| Available groEL gene sequences (HSP 60 kDa) |
|-------------------------------------------|
| **New Thailand isolates**                 |
| Species                        | Isolate | NCBI accession number | Strain | Country          | Year of isolation | **NCBI strains** |
|------------------------------- |---------|-----------------------|--------|-----------------|-------------------|-----------------|
| *Orientia tsutsugamushi*        | UT76    | EF551292              | Karp   | North-eastern Thailand | 2003             | *O. tsutsugamushi* |
|                              | UT125   | EF551293              | Gilliam| North-eastern Thailand | 2003             | *O. tsutsugamushi* |
|                              | UT144   | EF551294              | Gilliam| North-eastern Thailand | 2004             | *O. tsutsugamushi* |
|                              | UT150   | EF551295              | Karp   | North-eastern Thailand | 2004             | *O. tsutsugamushi* |
|                              | UT167   | EF551296              | Karp   | North-eastern Thailand | 2004             | *O. tsutsugamushi* |
|                              | UT169   | EF551297              | Karp   | North-eastern Thailand | 2004             | *O. tsutsugamushi* |
|                              | UT176   | EF551298              | Karp   | North-eastern Thailand | 2004             | *O. tsutsugamushi* |
|                              | UT177   | EF551299              | Karp   | North-eastern Thailand | 2004             | R. akari         |
|                              | UT196   | EF551300              | Gilliam| North-eastern Thailand | 2004             | R. prowazeki     |
|                              | UT213   | EF551301              | Karp   | North-eastern Thailand | 2004             | R. akani        |
|                              | UT219   | EF551302              | Karp   | North-eastern Thailand | 2004             | ATCC VR-148      |
|                              | UT221   | EF551303              | Karp   | North-eastern Thailand | 2004             | R. bedi         |
|                              | UT301   | EF551304              | Karp   | North-eastern Thailand | 2004             | R. canoni       |
|                              | UT302   | EF551305              | Karp   | North-eastern Thailand | 2004             | Malish          |
|                              | UT329   | EF551305              | Gilliam| North-eastern Thailand | 2004             | R. helvetica    |
|                              | UT332   | EF551306              | Karp   | North-eastern Thailand | 2004             | NS              |
|                              | UT333   | EF551307              | Karp   | North-eastern Thailand | 2004             | ATCC VR-1363    |
|                              | UT340   | EF551308              | Gilliam| North-eastern Thailand | 2004             | R. japonica     |
|                              | UT345   | EF551309              | Karp   | North-eastern Thailand | 2004             | AF342181        |
|                              | UT418   | EF551310              | Karp   | North-eastern Thailand | 2004             | R. rickettsi     |
|                              | UT419   | EF551311              | Karp   | North-eastern Thailand | 2004             | Botarret        |
|                              | UT48    | EF551312              | Karp   | North-eastern Thailand | 2004             | L96733          |
|                              | FPW2031 | EF551288              | TA716-like | Western Thailand | 2004             | R. sibirica     |
|                              | FPW2034 | EF551289              | Gilliam| Western Thailand | 2004             | ATCC VR-151     |
|                              | FPW2049 | EF551290              | Karp   | Western Thailand | 2004             | ATCC VR-148     |
|                              | FPW2049 | EF551291              | Gilliam| Western Thailand | 2004             | ATCC VR-151     |

| NCBI strains                  | Species | Strain              | NCBI accession number |
|-------------------------------|---------|---------------------|-----------------------|
| *O. tsutsugamushi*            | Boryong |                     | AY059015               |
| *O. tsutsugamushi*            | Karp    |                     | M31887                 |
| *O. tsutsugamushi*            | Gilliam |                     | AY191585               |
| *O. tsutsugamushi*            | Hwasung |                     | AY191589               |
| *O. tsutsugamushi*            | Kato    |                     | AY191586               |
| *O. tsutsugamushi*            | Kawasaki|                     | AY191587               |
| *O. tsutsugamushi*            | Youngworl |                 | AY191588               |
| *Rickettsia typhi*            | Wilmington |               | AY191590               |
| *R. prowazeki*                | Brai   |                     | Y15783                 |
| *R. akani*                   | ATCC VR-148 |             | AY059013               |
| *R. bedi*                    | RML369-C |                     | NC 007 940             |
| *R. canoni*                  | Malish |                     | AY059012               |
| *R. helvetica*               | NS     |                     | DQ442911               |
| *R. japonica*                | ATCC VR-1363 |            | AF342181               |
| *R. rickettsi*               | Botarret |                 | L96733                 |
| *R. sibirica*                | ATCC VR-151 |            | AY059014               |

NS, not specified; HSP, heat shock protein.
Alignments of the groEL gene sequences were used for primer design of the *O. tsutsugamushi* specific real-time PCR assay.
*Based on 56-kDa sequence typing.*
Conventional PCR

DNA was extracted with the Wizard SV Genomic DNA purification system (Promega, Madison, WI, USA). Amplification of the partial groEL gene was performed using PCR with the previously described [13] primers 5′-GTTGAAGTT/AGTTAAAGG-3′ (forward) and 5′-TTTTTTTTTTT/ATCATATAATCC-3′ (reverse), generating a product of 534–546 bp. A PCR reaction mix consisted of 50 ng of template DNA, 20 nmol of each primer, 1 U of Taq DNA polymerase, 1.5 mM MgCl₂ and distilled water in a total volume of 20 μL. Following 30 cycles of amplification (94°C/176°C, 30 s; 44°C, 45 s; and 72°C, 45 s) and a 5-min extension at 72°C on a thermocycler (PTC-200; Bio-Rad, Hercules, CA, USA), the PCR products were subjected to electrophoresis in agarose (2%, w/v) gel (Bio-Rad, Hercules, CA, USA). DNA sequencing was performed commercially by Macrogen, Seoul, South Korea, using BigDye™ terminator cycling conditions on an automated ABI model 3730XL nucleotide sequencer (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences were edited to equal length (459 bp), and alignments were performed using the Clustal W algorithm [17]. The resultant pairwise percentage divergence was calculated using Megalign software (DNASTAR Lasergene v6 package, DNASTAR, Inc., Madison, WI, USA). The derived O. tsutsugamushi groEL sequences were uploaded to GenBank (accession numbers EF551288–EF551310; see Table 1). The reference nucleotide sequences were downloaded from GenBank.

Real-time PCR

On the basis of alignments of sequences determined from conventional PCR products and sequences available from GenBank, a set of specific primers for the generation of a 160-bp amplicon of the groEL gene of O. tsutsugamushi was designed using PrimerSelect Version 6.1 software (DNASTar, USA); forward primer, 5′-TTGCAACRAATCGTGAAAAG-3′; and reverse primer, 5′-TCTCCGTCTACATCATCAGCA-3′. The PCR reaction mix contained primers at a final concentration of 200 nM each, 2 μL of DNA template, 10 μL of master mix (QuantiMix Easy, Biotools, Madrid, Spain) containing SYBR green, Taq polymerase, MgCl₂ (4 mM), dNTPs and distilled water in a final volume of 20 μL. The PCR reactions were performed and analysed using a Rotor-Gene 3000 (Corbett Research, Mortlake, NSW, Australia) real-time thermocycler, with an initial holding temperature of 95°C for 5 min, followed by 40 cycles at 95°C for 15 s, 54°C for 15 s and 72°C for 20 s, with fluorescence monitoring at the 54°C annealing step on a predetermined SYBR/FAM channel. Melting curve analysis was performed with increments of 1°C/step (72–95°C) to determine the change in peak fluorescence over time (dF/dT); positive results were confirmed by electrophoresis of the product in an agarose (3%, w/v) gel in TAE buffer and staining with ethidium bromide (BioRad, Hercules, CA, USA).

To determine detection limits of the assay, plasmids containing the amplified regions of groEL (O. tsutsugamushi UT176 strain) were generated by ligation into pGEM-T Easy Vectors (Promega, USA) and transferred by transformation into Escherichia coli, cultured overnight in a shaking incubator at 37°C in Luria Bertani broth and followed by plasmid extraction using the QIAprap Spin Miniprep Kit (Qiagen, Valencia, CA, USA).

The plasmids were purified and linearized by restriction enzyme digestion with pSTI (Promega, Madison, WI, USA). Linearized DNA was quantified using the Quant-IT PICO Green dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). Ten-fold dilution series were used as external controls, and the theoretical number of plasmid copies and corresponding reaction efficiencies were calculated (Rotor-Gene software, Rotor-Gene software Version 6.0 (Corbett Research, Australia).

**FIG. 1.** Graph depicting the dilution series (left diagram) of plasmids as external controls to create a standard curve (right diagram) for quantitative analysis. Plasmids are depicted as black ovals, and test samples as hollow circles, the top left circle representing the non-template controls (NTC). Results are per 2 μL of sample, with a limit of detection being 3 copies/μL for both plasmids and buffy coat samples. The copy numbers and corresponding reaction efficiencies were calculated using Rotor-Gene software Version 6.0 (Corbett Research, Australia).
Version 6.0; Corbett Research, Australia). Real-time PCR was performed with duplicates of each serial dilution to create a standard curve (Fig. 1).

The specificity of the real-time PCR was determined in three individual runs by assessment of rickettsial reference strains that were grown in VERO cell cultures. The reference strains included SFG members (R. conorii, Malish strain; Rickettsia rickettsii, Bitterroot strain; Rickettsia honei, RB strain; Rickettsia australis, JC strain; Rickettsia sibirica, 246 strain; Rickettsia akari, Kaplan strain), TG members (Rickettsia prowazekii, Breinl strain; R. typhi, Wilmington strain), and an ancestral group strain (Rickettsia bellii). Members of the orders Anaplasmataceae, Bartonellaceae and Coxillaceae were used as negative controls (Anaplasma phagocytophilum, Bartonella bacilliformis, Bartonella henselae, Bartonella vinsonii, Ehrlichia chaffeensis and Coxiella burnetii). DNA extracts from additional non-rickettsial bacteria (Burkholderia pseudomallei, E. coli, Enterococcus faecalis, Klebsiella pneumoniae and Salmonella enterica serovar Typhi) all yielded negative results.

Results

groEL sequence alignments

The 459-bp groEL sequences determined for all Thai isolates were compared with those of reference strains deposited in GenBank, to determine pairwise similarities of nucleotides and amino acids (Table 3; Fig. 2). The overall nucleotide identity for all available O. tsutsugamushi isolates, Thai and non-Thai strains, was 98.8% (range: 95.0–100%). The non-Thai O. tsutsugamushi reference strains (Karp, Kato, Gilliam, Boryong, Hwasung, Youngworl and Kawasaki) demonstrated a mean intragroup similarity of 96.3% (range: 95.0–100%). The mean nucleotide intragroup identity for Thai O. tsutsugamushi isolates was 99.5% (range: 98.9–100%) and the intra-subgroup identities were 99.7% for Karp and 99.6% for Gilliam group members respectively (Table 2, subgroup data not shown).

Comparison of percentage identities of groEL gene sequences among antigenic groups showed that Thai strains and non-Thai strains shared a mean nucleotide identity of 96.7%. When Thai O. tsutsugamushi strains were compared with TG and SFG strains, the percentage identity levels were found to be 67.5% and 65.6%, with similar values for non-Thai Orientia strains when compared to TG and SFG strains, i.e. 67.2% and 65.7%, respectively (Table 3).

Deduced groEL amino acid sequences

The Thai O. tsutsugamushi isolates also demonstrated a high overall mean intragroup identity of 98.6% in the amino acid sequences as compared to 89.9% for the non-Thai O. tsutsugamushi isolates (Table 2). Further subgroup analysis was based on the full open reading frame (ORF) sequences of the more variable 56-kD outer membrane protein derived from 23 isolates [18] and demonstrated a dominance of the Karp subtype in Thailand (15 of the total 23 isolates) with 99.7% average amino acid identity followed by the Gilliam subgroup (7/23) with 99.6% and the TA716-like group (1/23) with 98.9% amino acid identity (data not shown).

Comparison of deduced GroEL sequences of STG with those of TG and SFG isolates revealed low amino acid identity values of 28.6% and 29.2%, respectively (Table 3).

Real-time PCR

The assay repeatedly demonstrated a detection limit of < 3 copies/μL of O. tsutsugamushi, using serial dilutions of linearized plasmids (Fig. 1). Amplicons with appropriate melting temperatures (average, 84.6°C; range, 84.3–85.1°C) were produced from all O. tsutsugamushi plasmids, isolates and clinical samples. All templates derived from clinical isolates and strains belonging to the TG and SFG repeatedly and reliably led to negative results.

Quantitative data obtained with the clinical buffy coat samples, including two samples from Laotian patients, demonstrated copy numbers ranging from 2 to 31,668 copies/μL, with a median value of 64 copies/μL of buffy coat. These values represent bacterial loads in admission samples, corresponding to a median “days of fever” time of 6 (5–10) (interquartile range). One sample (UT530) was a clear outlier, both for buffy coat and isolation quantitation, as it demonstrated high bacterial loads in both samples; 28,237/μL of buffy coat and 1,059,061/μL of VERO cell culture at 100% infection of cells as determined by IFA. In cell culture sam-

![Fig 1](https://example.com/f1.png)

**TABLE 2. Mean percentage intragroup identity of all currently available groEL gene sequences within the scrub typhus group (STG), typhus group (TG) and spotted fever group (SFG)**

| Antigenic group | Nucleotides (range) | Amino acids (range) |
|-----------------|---------------------|---------------------|
| STG, Thai       | 99.5 (98.9–100.0)   | 98.6 (96.7–100.0)   |
| STG, non-Thai   | 96.3 (95.0–100.0)   | 89.9 (85.6–100.0)   |
| STG, all available groups | 98.8 (95.0–100.0)   | 96.7 (85.6–100.0)   |
| TG              | 96.7                | 91.5                |
| SFG (including Rickettsia bellii) | 92.8 (86.5–99.8)   | 81.1 (66.7–99.3)   |
| TG and SFG      | 92.5 (86.3–99.8)   | 82.3 (65.4–99.3)   |
| Overall STG, TG and SFG | 91.8 (64.0–100.0)   | 82.2 (26.8–100.0)   |

*Reported by Lee et al [13].

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ples, copy numbers ranged between $5.3 \times 10^3$ and $1.4 \times 10^6$ copies/µL (median $7.0 \times 10^4$) of DNA extract (Table 4).

**Discussion**

The results presented here demonstrate a high level of conservation among the groEL nucleotide and corresponding amino acid sequences of contemporary Thai and non-Thai reference isolates of *O. tsutsugamushi*. The high mean nucleotide intragroup identities among Thai *O. tsutsugamushi* isolates can be attributed to the similarities between the two main subgroups, Karp and Gilliam. The groEL gene is highly conserved but sufficiently variable to form the basis for genetic target design allowing differentiation of the genera *Orientia* and *Rickettsia*, as sequence analysis demonstrated 99.5% identity within the current 23 Thai isolates.
TABLE 3. Mean intergroup percentage identities of groEL nucleotide and GroEL amino acid sequences for scrub typhus group (STG), typhus group (TG) and spotted fever group (SFG) isolates

| Antigenic group | Isolates (n = 39) | STG | STG, non-Thai | STG, all isolates | TG | SFG | SFG, all isolates |
|-----------------|------------------|-----|---------------|------------------|----|-----|------------------|
| STG, Thai       | 23               | 96.7| 98.8          | 67.5             | 66.0|       |                  |
| STG, non-Thai   | 7                | 90.8| 96.3          | 67.2             | 65.7| 65.0| 66.0             |
| STG, all isolates | 30          | 94.5| 90.8          | 67.5             | 65.6| 66.0| 66.0             |
| TG              | 2                | 28.6| 29.3          | 28.7             | 91.4| 93.2|                  |
| SFG             | 7                | 29.2| 29.9          | 29.3             | 77.0| 92.4|                  |
| TG and SFG      | 9                | 29.1| 29.8          | 29.2             | 81.8| 81.1|                  |

The values depicted in the upper right section above the dividing diagonal represent nucleotide identities; those in the lower left section under the diagonal represent amino acid identities.

*Reported by Lee et al. [13].

and 98.8% identity for all available groEL sequences, including those deposited in GenBank. By comparison, a gene analysis based on sequences encoding the 56-kDa outer membrane protein, covering the full ORF of approximately 1600 bp, demonstrated only 80% identity within the same 23 isolates, underlining the high variability and limitations of this gene as a target for molecular diagnostic assays [18].

At present, only limited DNA sequence data covering the full ORF of the gene encoding the 47-kDa transmembrane protein of *O. tsutsugamushi* are available, but these data and preliminary sequencing results of strains across Asia (data not shown) are indicative of a high level of conservation.

Currently, three real-time assays are available for the detection of *O. tsutsugamushi*, targeting 16S rRNA genes [19], genes encoding the 47-kDa transmembrane protein [7] and the 56-kDa outer membrane protein [20].

To date, only the real-time PCR assay based on the 16S rRNA gene has been evaluated with a large number of clinical samples, and it has demonstrated a diagnostic sensitivity of 45%, using full blood samples drawn upon admission and IFA as a reference standard.

A recently described nested PCR assay showed an increased sensitivity of 82% [21], using buffy coat as a sample specimen. Both assays had excellent specificities and used reference standard IFA as a comparator, but with different cut-off levels for positivity. As *O. tsutsugamushi* is an obligate intracellular organism disseminating within white blood cells, this assay was based on buffy coat specimens, with the expectation of a concentration effect with higher bacterial loads. A prospective evaluation of the groEL-based real-time PCR assay presented here is underway.

On the basis of isolation and 16S rRNA gene sequencing, Karp and Kawasaki strains were found in the samples examined, and no Kato strains were included. Manosroi et al. [8] have previously described Karp and Kato strains in the same region, although this was based on a nested PCR assay with use of strain-specific nested primer sets. It is possible that these primers designed for the hypervariable 56-kDa protein-encoding gene could be less specific in detecting Gilliam strains in Thailand. In a recent study, the current group analysed the same 23 isolates by full open reading frame sequencing of the highly specific 56-kDa protein, and revealed the predominance of Karp strains and a substantial presence of Gilliam strains [18]. This new information adds to the existing groEL characterization data of five non-Thai isolates [13], suggesting that it is an ideal target for the development of molecular diagnostic assays for *O. tsutsugamushi*.

Stover et al. [22] first described the high degree of homology between the (formerly known) *Rickettsia tsutsugamushi* proteins Stp11 and Sta58 and the *E. coli* proteins GroES and GroEL, respectively, and the family of primor-

TABLE 4. Description and quantitation data (DNA copy numbers) from characterized clinical buffy coat samples based on the groEL real-time PCR assay

| Sample code | Days of fever | GroEL real-time PCR (Tm) | Quantitative real-time PCR (groEL copies present in 1 μL of buffy coat or tissue culture pellet) | 16S rRNA results | % identity | BLAST |
|-------------|---------------|--------------------------|-------------------------------------------------------------------------------------------------|------------------|-----------|-------|
| TC          | BC            | 1. TC | 2. TC | 3. TC | Average | 1. BC | 2. BC | 3. BC | Average | % identity | BLAST |
| UT512       | 6             | 84.5  | 85     | 655  | 092   | 252  | 376   | 321  | 738   | 409  | 735   | 34    | 90    | 44    | 56    | 99    | OT Karp      |
| UT528       | 6             | 84.7  | 84.5   | 81   | 500   | 56   | 200   | 49   | 642   | 62   | 447   | 30    | 6     | 6     | 14    | 99    | OT Karp      |
| UT530       | 3             | 84.3  | 84.4   | 96.1 | 32   | 86.1 | 052   | 13.50| 000   | 109.9| 061   | 31   | 668  | 23   | 009  | 30   | 034  | 28   | 237  | 99    | OT Kawasaki  |
| UT539       | 6             | 84.8  | 84.8   | 44   | 272   | 46   | 808   | 74   | 341   | 55   | 140   | 68    | 72   | 28   | 56    | 99    | OT Karp      |
| UT601       | 10            | 84.8  | 85.1   | 73   | 152   | 70   | 067   | 69   | 893   | 71   | 037   | 582   | 656  | 936  | 725   | 99    | OT Karp      |
| TM1055      | 5             | 84.7  | 84.7   | 37   | 416   | 39   | 467   | 33   | 126   | 36   | 670   | 4     | 3     | 4     | 4     | 99    | OT Kawasaki  |
| TM1084      | 10            | 84.5  | 84.5   | 930   | 9736  | 5319 | 7251  | 22   | 64    | 91   | 59    | 99    | OT Kawasaki |

*Tm* melting temperature of amplicon; TC, tissue culture; BC, buffy coat; OT, Orientia tsutsugamushi; UT, Udon Thani Hospital; TM, Mahosot Hospital, Vientiane, Laos PDR.
dial heat shock proteins designated Hsp10 and Hsp60. Although the sequence homology between the Sta58 antigen and the Hsp60 protein family was striking, the antigenic distinction among other bacterial Hsp60 homologues highlighted the uniqueness of this target, suggesting that it may be both a potentially protective antigen and a useful diagnostic reagent for scrub typhus. Park et al. [14,15] from Korea took further advantage of these features and incorporated the genetic information for identification, differentiation and characterization within the Rickettsiae and Anaplasmataceae. The resulting real-time PCR assay proved to be highly sensitive and specific for all tested isolates of O. tsutsugamushi. As scrub typhus is endemic in many resource-poor developing countries, groEL is a suitable candidate for the application of molecular methods in settings where the costs of establishing a real-time thermocycler are prohibitively high, but alternative methods, e.g. loop-amplified isothermal PCR, an accurate and relatively inexpensive technique, could be used [23,24]. In addition, the reliable and accurate quantitation of bacterial loads allows further investigation of other diagnostic methods and may lead to an improved understanding of the pathophysiology of this important neglected disease. Validation and evaluation in clinical settings in the field are underway.

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Transparency Declaration

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