Detection of Antibodies against *Orientia tsutsugamushi* Sca Proteins in Scrub Typhus Patients and Genetic Variation of *sca* Genes of Different Strains

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Scrub typhus, caused by *Orientia tsutsugamushi* infection, is one of the main causes of acute febrile illness in the Asian-Pacific region. Although early diagnosis and immediate antibiotic treatment are critical for reducing disease severity and mortality, current diagnostic methods using serological and molecular approaches have some limitations in sensitivity and applicability in clinical laboratories. In this study, we identified and characterized *O. tsutsugamushi* surface cell antigen (*sca*) family genes encoding autotransporter proteins in order to test them as novel diagnostic targets. We evaluated antibody responses against the Sca proteins in scrub typhus patient sera and examined the genetic diversity of these genes in different strains after PCR amplification. Specific antibody responses against ScaA and ScaC were observed in patients with high indirect immunofluorescence assay titers (>1:640), whereas specific responses against ScaB and ScaE were relatively low. Genetic analysis using genomic DNAs revealed the *sca* genes to be quite variable among the different strains. In contrast to *scaA*, *scaC*, and *scaD*, which were detected in all of the tested strains, *scaB* and *scaE* were amplified differentially from the different strains, suggesting a differential presence of the genes in the genomes. Among the members of the gene family, the sequence of *scaC* is the most highly conserved between the different strains, and the size of *scaD* is the most variable due to the presence of different numbers of internal repeat sequences. These results suggest that the *sca* genes of *O. tsutsugamushi* may be valuable targets for use in combination with classical assay methods for scrub typhus diagnosis.

Scrub typhus is an acute febrile illness caused by *Orientia tsutsugamushi* infection following the bite of an infected larval mite (9). It has been estimated that 1 billion people are at risk, and 1 million new cases arise annually in the Asian-Pacific region (23). This infectious disease has recently become an important public health issue due to regional outbreaks (16,18) and new emergence (6,28). Clinical presentations of scrub typhus, typically characterized by eschar, fever, rash, lymphadenopathy, and myalgia, can vary in severity from a mild and self-limiting flu-like syndrome to a life-threatening disease (13,25). Although early diagnosis and immediate antibiotic treatment are important to prevent severe complications of scrub typhus (27), the clinical discrimination of scrub typhus from other undifferentiated fevers, such as dengue and leptospirosis, is often very difficult because the clinical symptoms of these illnesses are similar (15,20). In addition, diagnosis of scrub typhus requires laboratory confirmation, usually by serologic detection of antibodies against the bacterial pathogen during the acute and convalescent phases of the disease, and the gold standard is the indirect immunofluorescence assay (IFA), which requires laborious bacterial culture in a biosafety level 3 facility (15). Moreover, despite their widespread use, all of the currently available serologic tests and PCR-based nucleic acid amplification methods have limitations which clinicians need to be aware of (15). For example, the bacterial antigen used for IFA is of high importance due to the risk of significant bias depending on the antigenic and genetic variation (14) of local strains in different regions of endemicity. The sensitivity of PCR-based nucleic acid amplification methods, which mainly target the gene for a major outer membrane protein, TSA56, has been reported to be as low as 50% (15). Although PCR targeting of the *tsa*56 gene has been shown to be highly specific, sequence variability may affect primer annealing and test sensitivity (15). Therefore, it has been proposed that new diagnostic assays using a panel of both serological and antigen detection systems targeting multiple antigens need to be developed to improve sensitivity (15,20).

Recently, our group reported that one gene (*scaC*) among a group of genes encoding autotransporter proteins in the *O. tsutsugamushi* genome is involved in bacterial adhesion to eukaryotic host cells, potentially through binding to host fibronectin (12). The *scaC* gene is highly conserved among different strains, and antibodies against the ScaC protein are detected in scrub typhus patients (12). *Rickettsia* species, comprising a sister group of *Orientia*, also possess multiple *sca* genes in their genomes (2). The rickettsial Sca proteins are involved in bacterial adhesion or the invasion process (3,24) and have been targeted for vaccine development (4). In order to discover whether the *sca* genes of *O. tsutsugamushi* can be used as novel diagnostic targets, we analyzed for the first time the antibody responses against diverse Sca proteins in scrub typhus patients and sequence variations of *sca* genes of different strains of *O. tsutsugamushi*.
MATERIALS AND METHODS

Ethics statements. Ethical approval for this work was granted by the institutional review boards of both Seoul National University Hospital (IRB no. 0-1001-039-307) and Chungnam National University Hospital (IRB no. 2008-10-08). All patients and healthy volunteers provided written informed consent prior to sample collection.

Patient samples. Human sera were collected during the scrub typhus endemic season (from September to January) from healthy volunteers.
and patients with acute febrile disease (n = 100) at Chungnam National University Hospital in Daejeon, Republic of Korea, after informed consent was obtained. All of the patients resided in the Chungcheong Province, in the middle part of the Republic of Korea, where the Boryong, Gilliam, and Karp strains are the most prevalent (5, 22). Primary diagnosis of scrub typhus was performed by IFA, and the *O. tsutsugamushi*-specific IFA titer was determined.

Preparation of *O. tsutsugamushi* and its genomic DNA. *O. tsutsugamushi* strains Boryong, Gilliam, Karp, and Kato were propagated in L929 cells (NTCT929; ATCC) and used for indirect immunofluorescence assays (see below) and the preparation of genomic DNA. *O. tsutsugamushi* was purified using a modification of a Percoll gradient purification method (17). At 3 to 4 days postinfection, infectivity was determined by IFA. When an infection rate of >90% was achieved, cells were harvested by centrifugation at 6,000 g for 20 min. The cell pellet was resuspended with 6.5 ml of Tris-sucrose (TS) buffer (33 mM Tris-Cl [pH 7.4] and 0.25 M sucrose). Resuspended cells were homogenized with a Polytron homogenizer (Wheaton Inc., Millville, NJ) for 100 strokes and centrifuged at 200 × g for 5 min. The supernatant was mixed with 40% Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) in TS buffer and centrifuged at 25,000 × g for 60 min. The bacterial band was collected and centrifuged at 77,000 × g for 30 min. *O. tsutsugamushi* was collected and used for genomic DNA extraction by use of an RBC genomic DNA extraction kit (RBC Bioscience Co., Taipei, Taiwan) according to the manufacturer’s instructions.

Cloning, expression, and purification of proteins. The *tsa56*, *p47*, and *sca* genes were PCR amplified from *O. tsutsugamushi* genomic DNA by use of the specific primer pairs presented in Table 1. The amplified region of each gene is summarized in Table 1 and Fig. 1A. The amplified DNAs were then cloned into a pET28a vector for sequence analysis and protein expression. All of the genes used for protein purification were derived from the *O. tsutsugamushi* Boryong strain, and the *sca* genes used for sequence analysis were amplified from the genomic DNAs of the Boryong, Gilliam, Karp, and Kato strains. *Escherichia coli* BL21(DE3) (Novagen, Darmstadt, Germany) was transformed with the recombinant plasmids for protein expression and purification. Protein expression was induced by adding 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Duchefa, Zwijndrecht, Netherlands) for 4 h at 37°C. Bacteria were harvested by centrifugation at 1,000 × g for 10 min, resuspended in binding buffer (300 mM NaCl, 50 mM sodium phosphate buffer, 10 mM imidazole) containing 1 mg/ml of lysozyme, incubated at 4°C for 30 min, and

**TABLE 2** Sensitivity and specificity of ELISAs using different *O. tsutsugamushi* antigens

| Antigen          | Sensitivity (%) | Specificity (%) |
|------------------|----------------|-----------------|
| TSA56            | 100            | 100             |
| 47-kDa protein   | 77             | 93              |
| ScaA             | 100            | 73              |
| ScaB             | 63             | 87              |
| ScaC             | 100            | 67              |
| ScaE             | 80             | 100             |
disrupted by sonication on ice for 5 min. The sonicated lysates were centrifuged at 1,600 \( \times \) g for 20 min at 4°C, and the supernatants were applied to a Ni-nitrilotriacetic acid (Ni-NTA) His-binding resin (Novagen) pre-equilibrated with binding buffer. His-tagged proteins bound to the Ni-NTA resin were eluted with elution buffer (300 mM NaCl, 50 mM sodium phosphate buffer, 250 mM imidazole) and serially dialyzed against elution buffer to remove any free imidazole. Finally, the identity and purity of the proteins were assessed by Western blotting and Coomassie blue staining, respectively.

**Immunofluorescence assay.** Immunofluorescence assay was used to measure antibody titers against *O. tsutsugamushi* (12, 14). L929 cells infected with each strain of *O. tsutsugamushi* (Boryong, Karp, or Gilliam) were harvested, mixed in equal amounts, and used as antigens for immunofluorescence assay. Infected L929 cells were harvested, washed with phosphate-buffered saline (PBS, pH 7.2), spotted onto Teflon-coated spot slides, and fixed with cold acetone for 10 min. The slides were stored at −70°C until used. Twofold serially diluted (1:40 to 1:1,280 in PBS) patient sera were added to the antigen-coated spot on the slide and incubated for 30 min in a moist chamber at room temperature. Alexa Fluor 488-conjugated goat anti-human IgG (Molecular Probes), diluted 1:1,000 in PBS, was used as the secondary antibody. The stained slides were examined under an Olympus FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan).

**ELISA.** All of the purified protein antigens used for enzyme-linked immunosorbent assay (ELISA) were derived from the Boryong strain, since it was reported that this strain covers approximately 80% of *O. tsutsugamushi* infections in the region of endemity (5). Immunoassay plates (96-well plates; Nunc, Rochester, NY) were coated overnight with 100 \( \mu \)l of purified His-tagged antigens at a concentration of 1 \( \mu \)g/ml in 0.05 M bicarbonate buffer (pH 9.5) and blocked with 3% bovine serum albumin (BSA) at room temperature for 1 h. Human sera from the patients and healthy volunteers were diluted in PBS containing 3% BSA and 0.1% Tween 20, added to the immunoassay wells, and incubated at 37°C for 1 h. After washing six times with PBS containing 0.05% Tween 20, an anti-human IgG–horseradish peroxidase (HRP) conjugate (Promega), di-

**FIG 3** Comparison of ELISA data obtained using the indicated antigens and IFA titers. The reactivities of the sera (10 samples per IFA titer) were assessed by ELISA and plotted against IFA titers. The cutoff values (red lines) were determined using IFA-negative sera from 10 healthy volunteers (mean plus 2 standard deviations). Correlations between ELISA results and IFA titers were assessed by nonlinear regression analysis (blue lines), and the \( r^2 \) values are shown.

**FIG 4** Genetic analysis of the *sca* genes of different strains of *O. tsutsugamushi*. (A) Purified *O. tsutsugamushi* genomes were used as templates, and *sca* genes were amplified by PCR using specific sets of primers (Table 1). KP, Karp; KT, Kato; GM, Gilliam; BR, Boryong. (B) Phylogenetic tree of the *sca* genes identified in the genomes of the indicated strains. The red inset box shows a phylogenetic tree for the *tsa56* genes of the indicated strains.
luted 1:10,000 in PBS containing 3% BSA and 0.1% Tween 20, was added and incubated for 1 h. TMB microwell peroxidase substrate solution (KPL, Gaithersburg, MD) was then added to develop color for 7 min, and the reaction was stopped by the addition of 1 M H₃PO₄ solution. Absorbance was measured at 450 nm using an ELISA plate reader (Beckman).

Sequence analysis and alignment. The passenger domains of O. tsutsugamushi sca genes amplified from genomic DNAs of different strains, including Boryong, Gilliam, Karp, and Kato, were sequenced (Macrogen Co., Seoul, Republic of Korea). We also used DNA sequence information for sca genes found in the genomes of O. tsutsugamushi strains Boryong (GenBank accession no. AM494475.1) (9) and Ikeda (GenBank accession no. AP008981.1) (12, 19). The sca gene sequences were aligned for phylogenetic analysis by use of ClustalW embedded within MegAlign software (DNAstar Inc., Madison, WI). The degrees of identity among the Sca protein sequences were determined using calculated alignment. The sequence alignment results for the translated passenger domains from the five different strains are presented in Fig. 5. Phylogenetic analyses of tsa56 genes were performed using sequences deposited in GenBank (accession no. CAM79668 for strain Boryong, ABF50384 for strain Gilliam, YP_001937637 for strain Ikeda, AAA26391 for strain Karp, and AAA26397 for strain Kato).

Statistical analysis. Statistical analyses were performed using the nonlinear regression module embedded in SigmaPlot (Jandel, San Rafael, CA).

Nucleotide sequence accession numbers. The passenger domains of the O. tsutsugamushi sca genes amplified from genomic DNAs of different strains were deposited in GenBank under accession no. JQ996623 to JQ996633.

FIG 5 Alignment of amino acid sequences of Sca proteins detected in the genomes of O. tsutsugamushi strains. The passenger domains of the genes identified in the indicated strains were compared. (A) ScaA; (B) ScaC; (C) ScaD; (D) ScaE. The conserved sequences are boxed, and the internal repeat sequences detected in ScaD are underlined with red and blue lines.
RESULTS

Detection of antibodies against Sca proteins in scrub typhus patients. In order to examine anti-Sca protein antibody responses in scrub typhus patients, we screened 100 sera from potential scrub typhus patients during the endemic season in the Republic of Korea. Based on an IFA using L929 cells infected with *O. tsutsugamushi*, 70 sera with various IFA titers (from 0 to 1,280) were randomly selected and tested in ELISAs using purified Sca proteins (Fig. 1B). IFA-negative sera from 10 healthy volunteers were used to determine the cutoff values. The cutoff value was defined as the mean optical density at 490 nm (OD490) plus 2 standard deviations for each experimental condition (11). To determine the optimal dilution of serum under our ELISA conditions, sera from 10 healthy volunteers, 10 IFA-negative patients, and 10 patients with scrub typhus (IFA titers of 1:1,280) were serially diluted (1:200 to 1:3,200) and examined by ELISAs. As shown in Fig. 2, ELISA signals were generally reduced as the sera were diluted, indicating that the antigen-antibody reactions were not saturated within the dilution range. The cutoff values, which indicate the presence of nonspecific antibodies in the sera, were relatively higher for ELISAs using the 47-kDa protein, ScaB, or ScaE as the antigen. The sensitivity and specificity of ELISAs using each antigen were determined and are summarized in Table 2. TSA56, ScaA, and ScaC ELISAs showed 100% sensitivity, whereas the ScaB ELISA showed the lowest sensitivity (63%). In terms of specificity, the TSA56 and ScaE ELISAs showed the highest (100%) specificity, in contrast to the ScaC ELISA, which showed 67% specificity.

Correlation of IFA titers with ELISA results obtained using different Sca antigens. Next, nonlinear regression analysis was performed to compare IFA titers of patient sera after 1:1,000 dilution in PBS with the OD490 values from ELISAs using different *O. tsutsugamushi* antigens (Fig. 3). The best correlation of ELISA data with IFA titers was observed when ScaA was used as the antigen, whereas the ScaB test showed the lowest correlation (*r*^2^ = 0.082). It is notable that the OD490 values from the ELISA using TSA56 as antigen were rapidly saturated for patient sera starting at a 1:40 IFA titer, although there was a relatively low correlation (*r*^2^ = 0.325) compared to those of ELISAs using ScaA or ScaC. These results suggest that specific antibody responses against TSA56 are induced most robustly in scrub typhus patients and that anti-ScaA and anti-ScaC antibody responses are significant only in patients with high IFA titers (≥1:640). In addition, specific antibody responses against the 47-kDa protein, ScaB, and ScaC are relatively weaker in scrub typhus patients.

Genetic variation of *sca* genes of different strains of *O. tsutsugamushi*. The differential correlation of IFA titers with ELISA results obtained using different *O. tsutsugamushi* antigens prompted us to examine the presence and variation in the passenger domains of the *sca* genes of the different strains of *O. tsutsugamushi*. By using specific primer sets for each gene (Table 1), we were able to amplify the *scaA*, *scaC*, and *scaD* genes from the genomes of three prototype strains (Karp, Kato, and Gilliam strains) as well as from the Boryong strain (Fig. 4A). Interestingly, the sizes of amplified *scaD* genes were different for all four strains. In the case of *scaB*, the gene was detected only in the Boryong strain. On the other hand, we could not amplify *scaE* from the Kato strain. When we compared the sequences of the amplified *sca* genes, each gene group formed specific clusters in a phylogenetic tree (Fig. 5 Continued on following page).
4B). However, nucleotide sequence distances among the strains did not show any consistency among the different Sca groups, i.e., the sequence variation among the strains showed a different pattern within each *sca* group. The identities of translated amino acid sequences (Fig. 5) among the *sca* genes of different strains are also quite variable: 65.8 to 81.8% for ScaA, 77.4 to 97.5% for ScaC, 69.7 to 93.8% for ScaD, and 62.8 to 85.3% for ScaE. ScaC proteins are the most conserved, and ScaE proteins show the most variation among the different strains. The size variation observed among *scaD* genes of different strains is due mainly to the presence of different numbers of internal repeat sequences (Fig. 5). There are two types of amino acid repeat sequences. One is comprised of around 70 amino acids that repeat five times in the ScaD protein of the Boryong strain and three times in that of the Gilliam strain. The other consists of 16 amino acids that were detected five times in the gene of the Boryong strain and once in that of the Gilliam strain. It is also interesting that the phylogenetic distribution of *scaC* genes of different strains was most similar to that of *tsa56*.

**FIG 5 continued**
genes (Fig. 4B), which have 81.5 to 90.9% amino acid sequence identity.

DISCUSSION

Diagnosis of scrub typhus is an important issue that determines the administration of rapid and proper antibiotic therapy in local clinics in regions of endemicity (15). However, all currently available serological tests and PCR-based detection methods have limitations in terms of sensitivity, costs, and convenience. Moreover, evaluation of diagnostic methods has been hampered because the current gold standard, IFA, is imperfect (15). Therefore, there is an urgent need for alternative diagnostic methods that use rapid and accurate point-of-care technologies that are readily available, especially for patients in the acute phase of scrub typhus. Recently, combinatorial detection methods consisting of rapid immunochromatographic techniques (ICT) and bacterial DNA amplification have been proposed (1, 20). Combinatorial diagnosis can achieve 50 to 67% sensitivity when applied to a robust reference comparator set, i.e., the scrub typhus infection criteria (STIC), comprised of four parameters: positive cell culture isolation, an admission IgM titer of ≥1:1,280 using the gold standard IFA, a 4-fold increase of IFA IgM titer, and/or a positive result in at least two of three PCR assays. Compared to single conventional detection techniques and methods, the combination of DNA- and antibody-based detection methods has increased sensitivity, with a minimal reduction of specificity, and has expanded the time frame of adequate diagnostic coverage to extend throughout the acute phase of scrub typhus (20).

Although the combinatorial method can improve the sensitivity of diagnosis during the acute phase, several limitations still remain. First, sensitivity is still less than 70% for applying the STIC to define scrub typhus with a high level of confidence. Second, PCR detection and rapid ICT are largely dependent on a molecular detection of the passenger domain of the gene family as molecular targets for scrub typhus diagnosis. Currently, PCR-based detection methods targeting tsa56, the 47-kDa protein gene, and groEL have been used for species-specific amplification (15, 21). The sensitivity of these DNA detection methods maximizes at around 40% (15, 20). Although targeting the 16S rRNA gene yields a higher sensitivity (37.5 to 52.3%) under real-world conditions (15), all of these DNA-based techniques require sequencing after PCR amplification in order to identify the specific strain that has infected the patient. In this report, we found that two of the sca genes, scaB and scaE, were ampliﬁed differentially in different strains, suggesting a differential presence of the genes in the genomes of different strains. In fact, the scaB gene was present in the Boryong strain but absent in the Ikeda strain when their complete genome sequences were examined (12). Therefore, the molecular detection of sca genes could provide direct clues about O. tsutsugamushi strains as well as the species, without sequencing. Furthermore, the size of the passenger domain of the scaD gene is highly variable among the different strains due to the different numbers of internal repeat sequences (Fig. 4A and 5). This characteristic of the gene can provide valuable information on the genotypes of O. tsutsugamushi without any sequencing. Currently, we have examined three prototype strains, the Boryong strain, and the Ikeda strain for sequence comparison. Considering the geographic distribution of various genotypic variants, further studies on the genetic variation of sca genes in a wide geographic area may facilitate the usage of this gene family as molecular targets for scrub typhus diagnosis.

Finally, we propose for the first time that a panel of serological and molecular detection methods using the sca gene family as a novel target, combined with classical assay methods, can provide rapid and cost-effective technologies for scrub typhus diagnosis as well as for epidemiologic studies.

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