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 *tsa56* 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*.

1442 cvi.asm.org
Clinical and Vaccine Immunology p. 1442–1451
September 2012 Volume 19 Number 9

TABLE 1 Primer sequences used in this study

Gene	Primer direction	Primer sequence[a]	Product size (bp) (amplified region [nt positions])[b]
tsa56	Forward	GGC<u>GGATCC</u>CCAGGATTTAGAGCAGAG	1,344 (253–1596)
	Reverse	CGG<u>TCGAC</u>GAAGTTATAGCGCACA	
p47	Forward	CGG<u>GGATCC</u>ATTAGTGTAAATAGTTTATCC	1,278 (121–1398)
	Reverse	CGG<u>TCGAC</u>CTTATTAATATTAGGTAAAGC	
scaA	Forward	CGG<u>GGATCC</u>TCATCTGCTAGAGGTGAGATG	1,169 (2371–3540)
	Reverse	CGG<u>TCGAC</u>ATCGCCTTTTAAACCCGGGTTACT	
	Forward	GGC<u>GGATCC</u>TTACTTGGAGATTATACTG	1,121 (2414–3534)
	Reverse	CGC<u>TCGAG</u>TTGTTTATTGATATTAGATCC	
scaB	Forward	GGC<u>GGATCC</u>AGTACAACTCAAAGGATATTAGG	1,047 (70–1116)
	Reverse	CGG<u>TCGAC</u>ACTACTACAAATGTTTGATCC	
scaC	Forward	GGC<u>GGATCC</u>AAAAGTATAACTCCAGAAAAGTG	600 (97–696)
	Reverse	CGG<u>TCGAC</u>GTTTAATTTAGCACGATTTAT	
	Forward	GGC<u>GGATCC</u>ATGTACCAATCTAAT	1,578 (1–1578)
	Reverse	CGG<u>TCGAC</u>AAAATTAGTTCCTATATG	
scaD	Forward	AA<u>GGATCC</u>CAATTAAGTGAGCGACTA	1,926 (136–2061)
	Reverse	CG<u>GAATTC</u>TACCGTAGCAACAGTAACAGC	
scaE	Forward	GGC<u>GGATCC</u>AATGTAAATGCACAGCCCAATAG	1,332 (91–1422)
	Reverse	CGG<u>TCGAC</u>CTGTACTGTTGCTATTTTAGA	
	Forward	GGC<u>GGATCC</u>AATGTAAATGCACAGCCCAA	1,291 (111–1401)
	Reverse	CGG<u>TCGAC</u>CTGTACTGTTGCTATTTTAGA	

[a] Restriction enzyme sites are underlined.
[b] For Boryong strain genes.

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
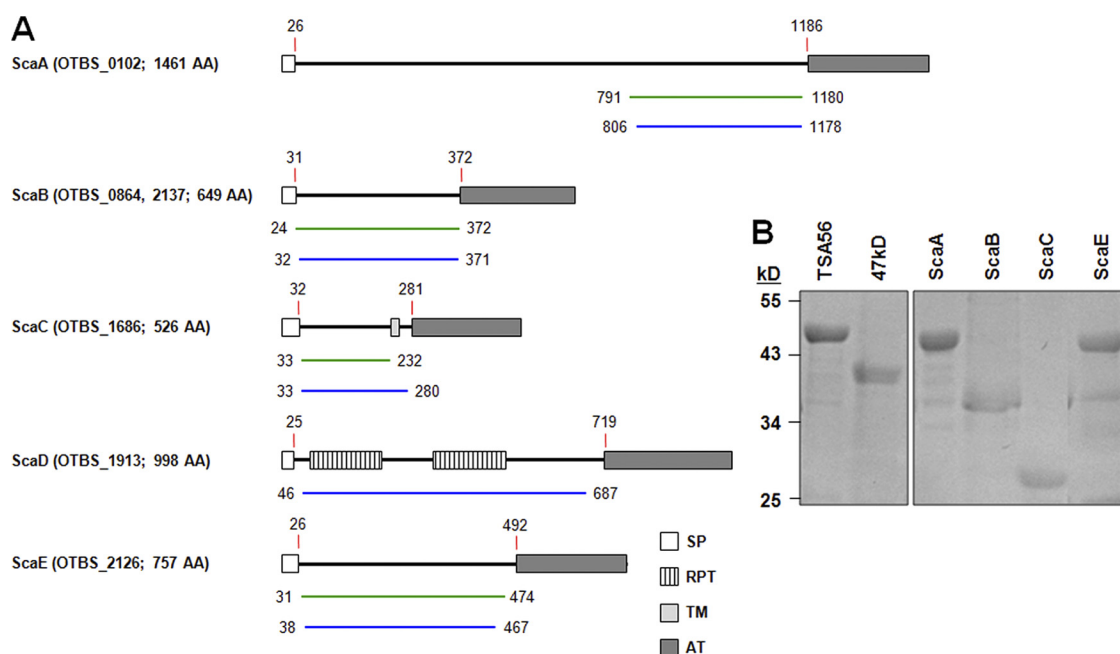
FIG 1 Schematic representation of the five Sca proteins whose genes were identified in the *O. tsutsugamushi* genome and demonstration of purified bacterial antigens. (A) Domain structures were predicted using SMART (simple modular architecture research tool [http://smart.embl-heidelberg.de/]). The amino acid positions (indicated by numbers) are based on the sequences from the Boryong strain. The green lines indicate the cloned region for protein expression, and the blue lines show the passenger domains used for sequence analysis after PCR amplification. SP, signal peptide; RPT, internal repeat sequence; TM, transmembrane domain; AT, autotransporter domain; AA, amino acids. (B) The bacterial antigens were cloned into the pET28a expression vector by use of the specific primers summarized in Table 1 and were expressed in *E. coli*. The His-tagged proteins were purified using Ni-NTA His-binding resin and then visualized by SDS-PAGE and Coomassie brilliant blue staining.
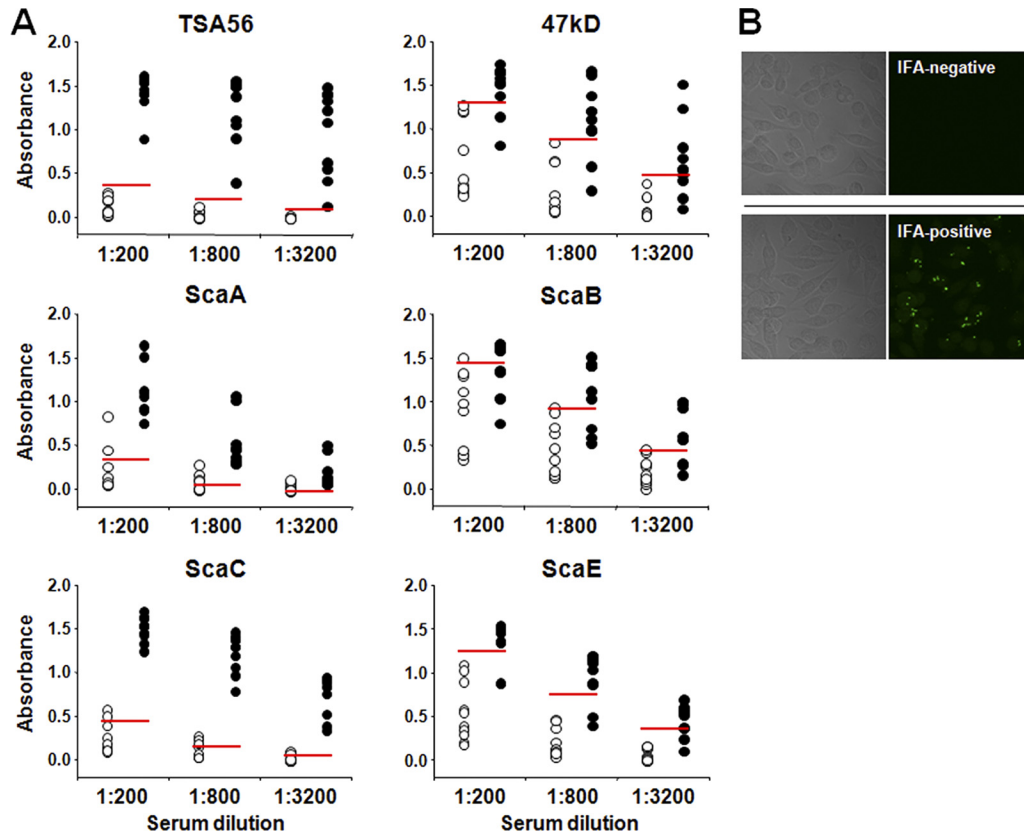
FIG 2 Antibody responses against the indicated *O. tsutsugamushi* antigens were analyzed by IgG ELISA. (A) Sera from 10 scrub typhus patients (filled circles; IFA titers of ≥1:1,280) and IFA-negative sera from 10 patients with acute febrile illness (open circles) were diluted as indicated and used for ELISAs. IFA-negative sera from 10 healthy volunteers were used to determine the cutoff values (red lines; mean plus 2 standard deviations). (B) Representative IFA results for a negative serum from a patient with acute febrile illness (top) and a positive serum from a scrub typhus patient (bottom). Differential interference contrast images of *O. tsutsugamushi*-infected cells are shown in the left panels.

($n = 10$) 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 $6,000 \times g$ 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 $200 \times g$ for 5 min. The supernatant was mixed with 40% Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) in TS buffer and centrifuged at $25,000 \times g$ for 60 min. The bacterial band was collected and centrifuged at $77,000 \times g$ 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 $1,000 \times g$ 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
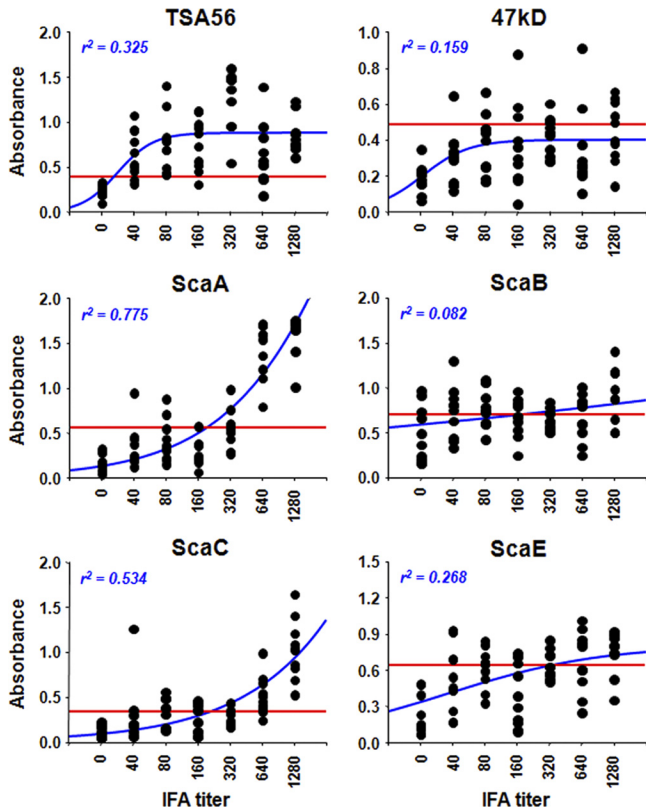
FIG 3 Comparison of ELISA data obtained using the indicated antigens and IFA titers. The reactivities of the sera (10 samples per IFA titer) were assessed by ELISA and plotted against IFA titers. The cutoff values (red lines) were determined using IFA-negative sera from 10 healthy volunteers (mean plus 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.

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) preequilibrated 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 endemicity (5). Immunoassay plates (96-well plates; Nunc, Rochester, NY) were coated overnight with 100 μl of purified His-tagged antigens at a concentration of 1 μg/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-
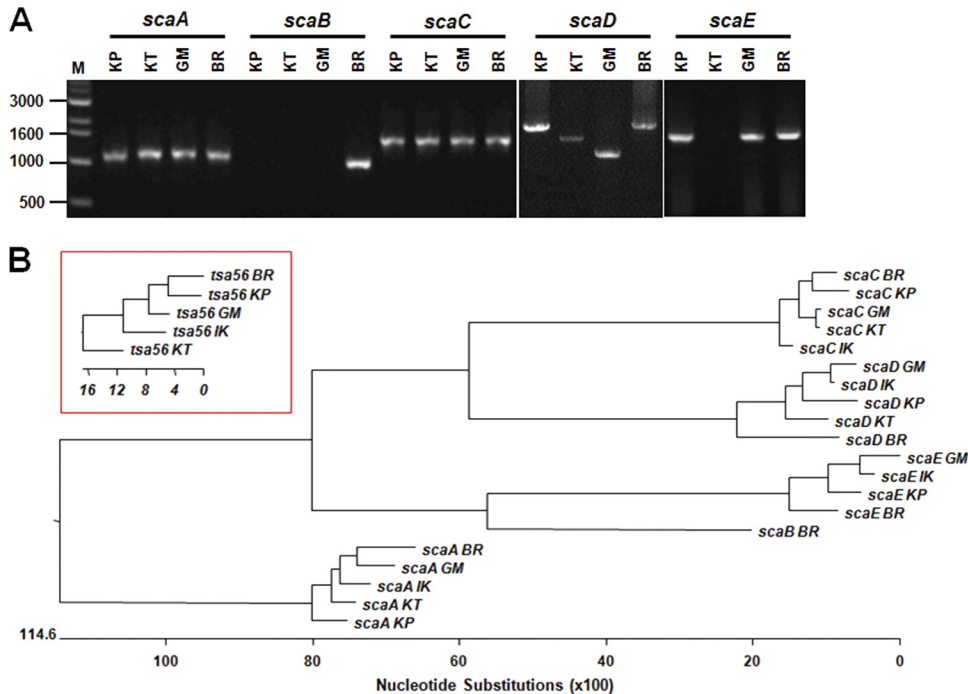
FIG 4 Genetic analysis of the *sca* genes of different strains of *O. tsutsugamushi*. (A) Purified *O. tsutsugamushi* genomes were used as templates, and *sca* genes were amplified by PCR using specific sets of primers (Table 1). KP, Karp; KT, Kato; GM, Gilliam; BR, Boryong. (B) Phylogenetic tree of 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.

A

(Figure: sequence alignment of ScaA amino acid sequences for strains BR, GM, IK, KP, and KT, with consensus rows and position numbering from 10 to ~370.)
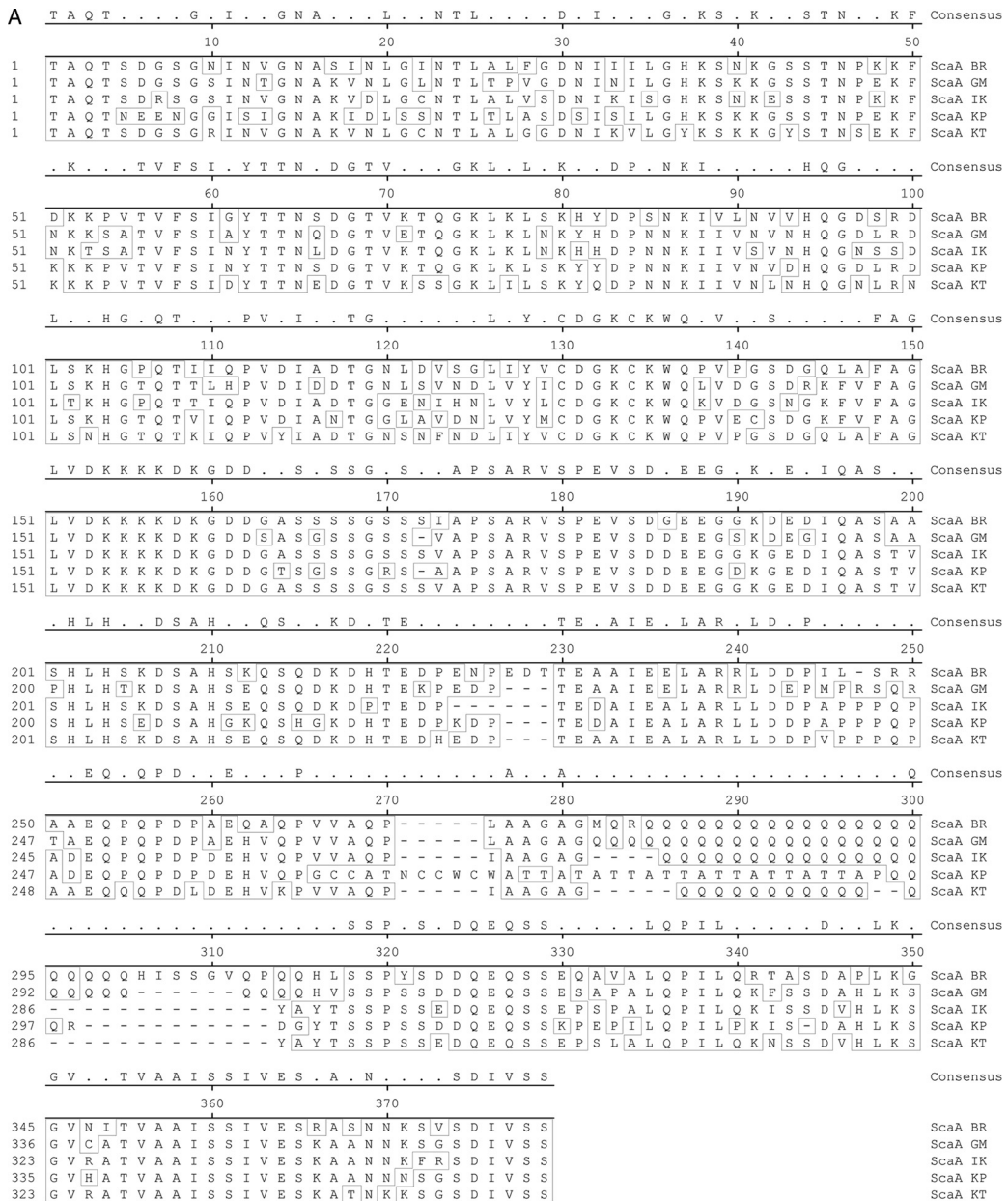
FIG 5 Alignment of amino acid sequences of Sca proteins detected in the genomes of *O. tsutsugamushi* strains. The passenger domains of the genes identified in the indicated strains were compared. (A) ScaA; (B) ScaC; (C) ScaD; (D) ScaE. The conserved sequences are boxed, and the internal repeat sequences detected in ScaD are underlined with red and blue lines.

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 H_3PO_4 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.

B

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        K S I T P E K . K . P Q A E . I . . . E D E E . . . . . . . . . E . A D N . L . . . V S N M N L D I S   Consensus
                  10              20              30              40              50
  1   K S I T P E K C K Y P Q A E D I Y E E D E E D E E D E E D E E E I A D N D L L N V V S N M N L D I S   ScaC BR
  1   K S I T P E K G K Y P Q A E D I H K E D E E - - - - - - - - - E I A D N N L L N V V S N M N L D I S   ScaC GM
  1   K S I T P E K G K Y P Q A E D I H K E D E E - - - - - - - - - E I A D N G L L D A V S N M N L D I S   ScaC IK
  1   K S I T P E K G K C P Q A E N I H K E D E E - - - - - - - - - E V A D N D L S N V V S N M N L D I S   ScaC KP
  1   K S I T P E K G K Y P Q A E D I H K E D E E - - - - - - - - - E I A D N D L L N V V S N M N L D I S   ScaC KT

        . . G . A R E G L R K D I . E . S K L L . . L E . S T . S S K N L T M R L Q Q . . . R . . E Q S . . .   Consensus
                  60              70              80              90              100
 51   D M G E A R E G L R K D I A E V S K L L C L L E Q S T A S S K N L T M R L Q Q S D I R E T E Q S N Y   ScaC BR
 42   N M G E A R E G L R K D I A E V S K L L C M L E K S T A S S K N L T M R L Q Q S D T R E N E Q S N H   ScaC GM
 45   G I G K A R E G L R K D I T E I S K L L Y M L E Q S T A S S K N L T M R L Q Q A N T R E N E Q S N Y   ScaC IK
 42   N M G E A R E G L R K D I A E V S K L L C M L E Q S T T S S K N L T M R L Q Q S N T R A T E Q S D Y   ScaC KP
 42   N M G E A R E G L R K D I A E V S K L L C M L E K S T A S S K N L T M R L Q Q S D T R E N E Q S N H   ScaC KT

        . . L L I T E I E S . N K E N . . L . Q . N N Q L K I K L . Q L . . . K . E Q . L A . . . T D L . . I L   Consensus
                  110             120             130             140             150
101   T G L L I T E I E S I N K E N N D L Q Q T N N Q L K I K L Q Q L E V K L E Q A L A R N T D L R Q I L   ScaC BR
 92   I R L L I T E I E S I N K E N D C L R Q K N N Q L K I K L H Q L K V K L E Q A L A R S T D L Q Q I L   ScaC GM
 95   T G L L I T E I E S I N K E N D Y L Q Q K N N Q L K I K L Q Q L E V K V E Q A L A R N T D L R Q I L   ScaC IK
 92   I R L L I T E I E S L N K E N D Y L Q Q K N N Q L K I K L Q Q L E A K L E Q V L A K N T D L Q P I L   ScaC KP
 92   I R L L I T E I E S I N K E N D C L R Q K N N Q L K I K L H Q L K V K L E Q A L A R N T D L Q Q I L   ScaC KT

        . . . . . A E . D . L . . Q N E Q L K . Q L Q Q L Q . . . L Q . . . . . L Q . K . . . N . . . . I N . . . K L N   Consensus
                  160             170             180             190             200
151   P E T R A E M D N L R E Q N E Q L K I Q L Q Q L Q V K L Q N L M S L Q N K D S N S V L I N R A K L N   ScaC BR
142   P K S Q A E M D N L W K Q N E Q L K I Q L Q Q L Q V K L Q N L M S L Q N K G S N L A L I N S V K L N   ScaC GM
145   L K P Q A E M D N L R K Q N E Q L K M Q L Q Q L Q L K L Q N L I S L Q D K D S N L A P I N G I K L N   ScaC IK
142   P E T R A E I D D L R K Q N E Q L K I Q L Q Q L Q V T L Q N S L F L Q N K N N P A L I N S V K L N   ScaC KP
142   P K S Q A E M D N L R K Q N E Q L K I Q L Q Q L Q V K L Q D L M S L Q N K C S N L A L I N S V K L N   ScaC KT

        Y I . D . K . I A L A N . V I V K A L A . T F . . K L S K S . R . K . H . . . I T . S G . N I D K   Consensus
                  210             220             230             240
201   Y I S D V K Y I A L A N I V I V K A L A G T F T D K L S K S N R I K K H C Y I T S S G S N I D K   ScaC BR
192   Y I R D I K Y I A L A N I V I V K A L A S T F T E K L S K S N R I K K H R H I T G S G S N I D K   ScaC GM
195   Y I N D V K C I A L A N T V I V K A L A C T F T D K L S K S N R I K N H C H I T S S G S N I D K   ScaC IK
192   Y I S D V K Y I A L A N I V I V K A L A G T F S D K L S K S H R I K K H C H I T S S G L N I D K   ScaC KP
192   Y I R D I K Y I A L A N I V I V K A L A S T F T E K L S K S N R V K K H R H I T G S G S N I D K   ScaC KT
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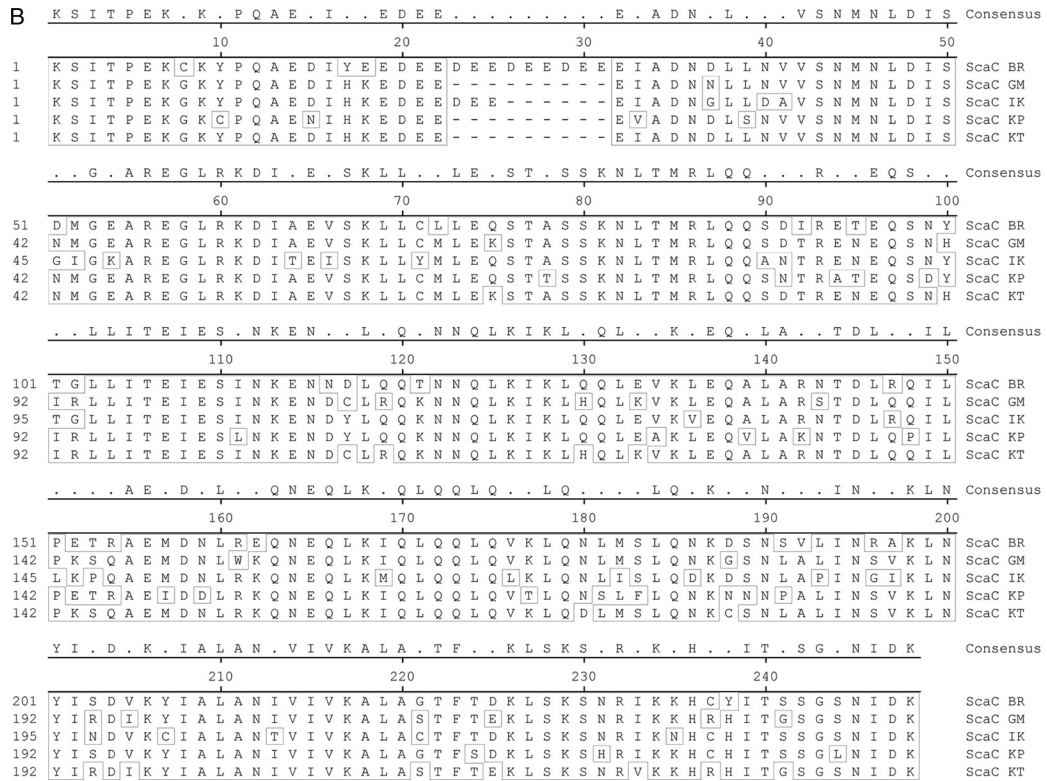
FIG 5 Continued on following page

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 (OD_{490}) 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 ≥1,280) were serially diluted (1:200 to ~1:3,200) and examined by ELISAs using the different antigens. 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-kD 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 OD_{490} values from ELISAs using different *O. tsutsugamushi* antigens (Fig. 3). The best correlation of ELISA data with IFA titers was observed when ScaA ($r^2 = 0.775$) was used as the antigen, whereas the ScaB test showed the lowest correlation ($r^2 = 0.082$). It is notable that the OD_{490} 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 ScaE 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.
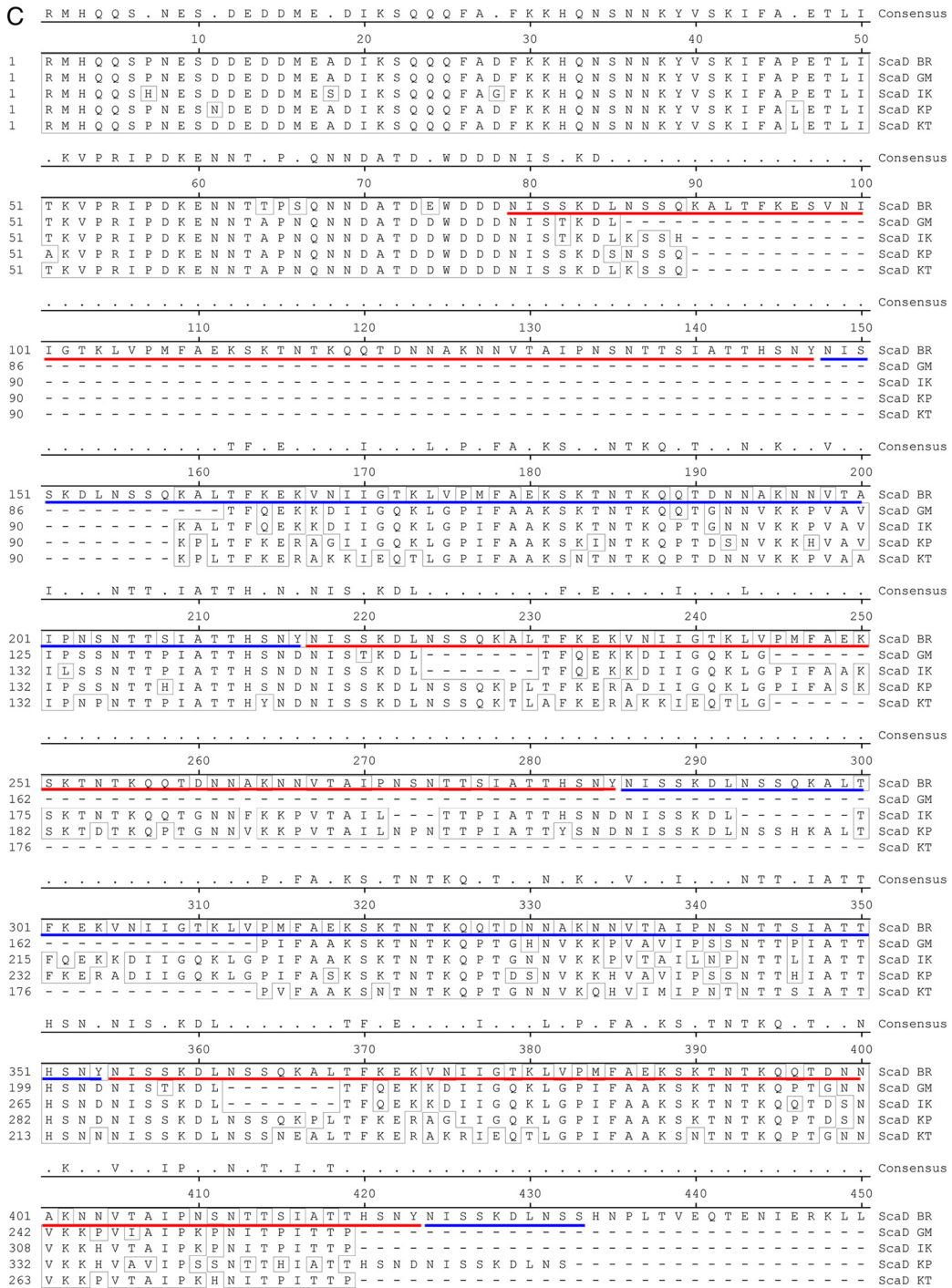
C

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        R M H Q Q S . N E S . D E D D M E . D I K S Q Q Q F A . F K K H Q N S N N K Y V S K I F A . E T L I   Consensus
                10          20          30          40          50
   1    R M H Q Q S P N E S D D E D D M E A D I K S Q Q Q F A D F K K H Q N S N N K Y V S K I F A P E T L I   ScaD BR
   1    R M H Q Q S P N E S D D E D D M E A D I K S Q Q Q F A D F K K H Q N S N N K Y V S K I F A P E T L I   ScaD GM
   1    R M H Q Q S H N E S D D E D D M E S D I K S Q Q Q F A G F K K H Q N S N N K Y V S K I F A P E T L I   ScaD IK
   1    R M H Q Q S P N E S N D E D D M E A D I K S Q Q Q F A D F K K H Q N S N N K Y V S K I F A L E T L I   ScaD KP
   1    R M H Q Q S P N E S D D E D D M E A D I K S Q Q Q F A D F K K H Q N S N N K Y V S K I F A L E T L I   ScaD KT

        . K V P R I P D K E N N T . P . Q N N D A T D . W D D D N I S . K D . . . . . . . . . . . . . . . .   Consensus
                60          70          80          90          100
  51    T K V P R I P D K E N N T T P S Q N N D A T D E W D D D N I S S K D L N S S Q K A L T F K E S V N I   ScaD BR
  51    T K V P R I P D K E N N T A P N Q N N D A T D D W D D D N I S T K D L - - - - - - - - - - - - - - -   ScaD GM
  51    T K V P R I P D K E N N T A P N Q N N D A T D D W D D D N I S T K D L K S S H - - - - - - - - - - -   ScaD IK
  51    A K V P R I P D K E N N T A P N Q N N D A T D D W D D D N I S S K D S N S S Q - - - - - - - - - - -   ScaD KP
  51    T K V P R I P D K E N N T A P N Q N N D A T D D W D D D N I S S K D L K S S Q - - - - - - - - - - -   ScaD KT

        . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   Consensus
                110         120         130         140         150
 101    I G T K L V P M F A E K S K T N T K Q Q T D N N A K N N V T A I P N S N T T S I A T T H S N Y N I S   ScaD BR
  86    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   ScaD GM
  90    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   ScaD IK
  90    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   ScaD KP
  90    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   ScaD KT

        . . . . . . . . . . . . . . . . . T F . E . . . . I . . . L . P . F A . K S . . N T K Q . T . . . N . K . . . V . . .   Consensus
                160         170         180         190         200
 151    S K D L N S S Q K A L T F K E K V N I I G T K L V P M F A E K S K T N T K Q Q T D N N A K N N V T A   ScaD BR
  86    - - - - - - - - - T F Q E K K D I I G Q K L G P I F A A K S K T N T K Q Q T G N N V K K P V A V   ScaD GM
  90    - - - - - - - - - K A L T F Q E K K D I I G Q K L G P I F A A K S K T N T K Q P T G N N V K K P V A V   ScaD IK
  90    - - - - - - - - - K P L T F K E R A G I I G Q K L G P I F A A K S K I N T K Q P T D S N V K K H V A V   ScaD KP
  90    - - - - - - - - - K P L T F K E R A K K I E Q T L G P I F A A K S N T N T K Q P T D N N V K K P V A A   ScaD KT

        I . . . . N T T . I A T T H . N . N I S . K D L . . . . . . . . . F . E . . . . I . . . L . . . . . . . .   Consensus
                210         220         230         240         250
 201    I P N S N T T S I A T T H S N Y N I S S K D L N S S Q K A L T F K E K V N I I G T K L V P M F A E K   ScaD BR
 125    I P S S N T T P I A T T H S N D N I S T K D L - - - - - - - T F Q E K K D I I G Q K L G - - - - -   ScaD GM
 132    I L S S N T T P I A T T H S N D N I S S K D L - - - - - - - T F Q E K K D I I G Q K L G P I F A A K   ScaD IK
 132    I P S S N T T H I A T T H S N D N I S S K D L N S S Q K P L T F K E R A D I I G Q K L G P I F A S K   ScaD KP
 132    I P N P N T T P I A T T H Y N D N I S S K D L N S S Q K T L A F K E R A K K I E Q T L G - - - - -   ScaD KT

        . . . . . . . . . . . . . . . . . . . . . . . . P . F A . K S . T N T K Q . T . . . N . K . . V . . . I . . . N T T . I A T T   Consensus
                260         270         280         290         300
 251    S K T N T K Q Q T D N N A K N N V T A I P N S N T T S I A T T H S N Y N I S S K D L N S S Q K A L T   ScaD BR
 162    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   ScaD GM
 175    S K T N T K Q Q T G N N F K K P V T A I L - - - T T P I A T T H S N D N I S S K D L - - - - - - - T   ScaD IK
 182    S K T D T K Q P T G N N V K K P V T A I L N P N T T P I A T T Y S N D N I S S K D L N S S H K A L T   ScaD KP
 176    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   ScaD KT

        H S N . N I S . K D L . . . . . . . . . T F . E . . . . I . . . L . P . F A . K S . T N T K Q . T . . . N   Consensus
                310         320         330         340         350
 301    F K E K V N I I G T K L V P M F A E K S K T N T K Q Q T D N N A K N N V T A I P N S N T T S I A T T   ScaD BR
 162    - - - - - - - - - - - P I F A A K S K T N T K Q P T G H N V K K P V A V I P S S N T T P I A T T   ScaD GM
 215    F Q E K K D I I G Q K L G P I F A A K S K T N T K Q P T G N N V K K P V T A I L N P N T T L I A T T   ScaD IK
 232    F K E R A D I I G Q K L G P I F A S K S K T N T K Q P T D S N V K K H V A V I P S S N T T H I A T T   ScaD KP
 176    - - - - - - - - - - - P V F A A K S N T N T K Q P T G N N V K Q H V I M I P N T N T T S I A T T   ScaD KT

        . K . . . V . . . I P . . N . T . I . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   Consensus
                360         370         380         390         400
 351    H S N Y N I S S K D L N S S Q K A L T F K E K V N I I G T K L V P M F A E K S K T N T K Q Q T D N N   ScaD BR
 199    H S N D N I S T K D L - - - - - - - T F Q E K K D I I G Q K L G P I F A A K S K T N T K Q P T G N N   ScaD GM
 265    H S N D N I S S K D L - - - - - - - T F Q E K K D I I G Q K L G P I F A A K S K T N T K Q Q T D S N   ScaD IK
 282    H S N D N I S S K D L N S S Q K P L T F K E R A G I I G Q K L G P I F A A K S K T N T K Q P T D S N   ScaD KP
 213    H S N N N I S S K D L N S S N E A L T F K E R A K R I E Q T L G P I F A A K S N T N T K Q P T G N N   ScaD KT

        . K . . . V . . . I P . . N . T . I . T . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   Consensus
                410         420         430         440         450
 401    A K N N V T A I P N S N T T S I A T T H S N Y N I S S K D L N S S H N P L T V E Q T E N I E R K L L   ScaD BR
 242    V K K P V I A I P K P N I T P I T T P - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   ScaD GM
 308    V K K H V T A I P K P N I T P I T T P - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   ScaD IK
 332    V K K H V A V I P S S N T T H I A T T H S N D N I S S K D L N S - - - - - - - - - - - - - - - - -   ScaD KP
 263    V K K P V T A I P K H N I T P I T T P - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   ScaD KT
```

FIG 5 continued

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*

Panel C

```
                              L                                              Consensus
              460       470       480       490       500
451 P I I S K T A A E L K N I N T K L T L I C S D Q S N A E I D D I - - Y H D A E Y N Q S N A E I D D I  ScaD BR
261 - - - - - - - - - - T H Q T L I Y S G Q S - - - - - - - - - - - - - - - - - - - - - - - -          ScaD GM
327 - - - - - - - - - - T H Q T L I C S G Q S N A E A E D I - - Y Y D A E D N Q S - - - - - -            ScaD IK
364 - - - - - - - - - - S Q K P L T F K E R A D K I E Q K L G P I F A A K S K T N - - - - - -            ScaD KP
282 - - - - - - - - - - T H Q T L I C S D Q S N D E I E D I - - C Y D A E D V L - - - - - - -            ScaD KT

                                                          N                                  Consensus
              510       520       530       540       550
499 Y H D A E Y N Q S N A E I D D I Y H D A E Y N Q S N A E I D D I Y H D A E Y N Q S N A E I D D I Y H  ScaD BR
272 - - - - - - - - - - - - - - - - - - - - - - - - - N V A T E D I Y Y D A E D N Q S N A A T E D I Y Y  ScaD GM
354 - - - - - - - - - - - - - - - - - - - - - - - - N A A T E D I Y Y D A E D N Q S N A A T E D I Y Y    ScaD IK
393 - - - - - - - - - - - - - - - - - - - - - - - - T K Q P T D N N V K K P V T A I P N P N T T P I T T  ScaD KP
308 - - - - - - - - - - - - - - - - - - - - - - - - D C S A N Q P S L L P P S T L N N S A V Q P L N N    ScaD KT

                          I     E D V L D C S . N Q P S L L . P S T L N N S A V Q P L      Consensus
              560       570       580       590       600
549 D A E Y - - - - - N Q S N A E I D D I Y H D A E D V L D C S T N Q P S L L L P S T L N N S A V Q P L  ScaD BR
297 D A E D - - - - - N Q S N A A T E D I Y Y D A E D V L D C S A N Q P S L L P P S T L N N S A V Q P L  ScaD GM
379 D A E D - - - - - N Q S N S A T E D I Y Y D A E D V L D C S A N Q P S L L P P S T L N N S A V Q P L  ScaD IK
419 P T H Q T L I C S G Q S N A A T E D I Y Y D A E D V L D C S A N Q P S L L P P S T L N N S A V Q P L  ScaD KP
333 N N Q Q - - - - - F D N S E Q N K T I E S E I E D V L D C S A N Q P S L L P S T L N N S A V Q P L    ScaD KT

    N N N N Q Q . D N S E Q N K T I E S E . K V . . . Y F K E Q E K . L L K A A Q T N . I A . T S A .   Consensus
              610       620       630       640
594 N N N N Q Q F D N S E Q N K T I E S E L K V K E Y F K E Q E K V L L K A A Q T N E I A T T S A I A  ScaD BR
342 N N N N Q Q F D N S E Q N K T I E S E I K V K A Y F K E Q E K V L L K A A Q T N D I A T T S A V    ScaD GM
424 N N N N Q Q L D N S E Q N K T I E S E I K V K A Y F K E Q E K V L L K A A Q T N D I A T T S A I I  ScaD IK
469 N N N N Q Q F D N S E Q N K T I E S E I K V R A Y F K E Q E K V L L K A A Q T N D I A T T S A I A  ScaD KP
378 N N N N Q Q F D N S E Q N K T I E S E I K V K A Y F K E Q E K A L L K A A Q T N D I A I T S A I A  ScaD KT
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Panel D

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    S T K Q . Q L . . . . . . . A . K . . L . . . Q Q . N L E . D R . K I Y N . . . . . . . . . I . L E   Consensus
              10        20        30        40        50
1 S T K Q N Q L N N K I N Q I D A R K S Q L L L Q Q Q N L E Q D R D K I Y N S I R D K I Y N G I N L E  ScaE BR
1 S T K Q D Q L N N K I N Q I D A R K F E L V Q Q Q N L E R D R E K I Y N - - - - - - - - R I D L E    ScaE GM
1 S T K Q D Q L Y - - - - - - - A T K S Q L L Q Q Q N L E R D R E K I Y N - - - - - - - - R I D L E    ScaE IK
1 S T K Q D Q L N N K I N Q I D A R K F E L V Q Q Q R N L E R D R E K I Y N - - - - - - - - R I D L E    ScaE KP

    . I . S . S . . . . . . L L . P S . Q V T V Y L . . . D . . . D . . . . . R . R S . M L . . H I . . .   Consensus
              60        70        80        90        100
51 D I K S Y S D V C R K L L L H P S D Q V T V Y L D E H D Q Y D T H - N L R P R S N M L Y R H I N E T  ScaE BR
43 K I R S H S D I L E Q L L L H P S E Q V T V Y L N K Q D P H D K N - Y V R P R S N M L H R H I N D A  ScaE GM
36 K I K S H S N I L D K L L L Y P S N Q V T V Y L N E Q D P H D K N - Y V R P R S N M L Y R H I N D A  ScaE IK
43 K I K S H S D I L D K F L L H P S N Q V T V Y L N E E D P H D R K K N T R T R S R M L Y K H I Q D T  ScaE KP

    . . . I . . M . . . . . . D I V . . Y T . . . . . . . . . A . L . . M D . . . . . . . . . V . R   Consensus
              110       120       130       140       150
100 A N F I N N M L G L I D I D I V P N Y T N R - - - - - - S A T L A E M K D Y E K Q L S D - - K V N R  ScaE BR
92 A N Y I N N M C T L I - V D I V P G Y T N N G P N A P D P A R L I T M T D Q K A Q L S N - - K V K R  ScaE GM
85 A N Y I N N M C T L M - V D I V P G Y T S N G P N A T D P A R L V T M T D Q E A Q L N S - - K V Q R  ScaE IK
93 K K L I D K M R P S I - V D I V S S Y T N N G P D A P D P A R L I T M T D Q E A K F N D N D R V A R  ScaE KP

    F . . R . . G . V N N . . . . . . R . V T . E I . E L . Q . . . . . . Q F H . A L . . V Q . . I . A L   Consensus
              160       170       180       190       200
142 F G N R I H G G V N N A K Q R Y R A V T I E I D E L N Q N Q E - L R Q F H T A L R D V Q A K I Q A L  ScaE BR
139 F G N R I R G - V N N A N Q Q C R A V T T E I N E L S Q A L N - - - Q F H Q A L G N V Q A E I Q A L  ScaE GM
132 F A N R L R G - V N N A K Q Q C R G V T T E I N E L S Q E L N - - - Q F H Q A L V N V Q A Q I Q A L  ScaE IK
142 F D D R I H G - V N N P G K K W R A V T T E I N E L S Q E L D K V F Q F H Q A L V D V Q N Q I K A L  ScaE KP

    S L . S L N D L . T E . . . . L H L . I . . Q L . . . D . . I . . C E . G S N S M L . L . . Q Y E Q .   Consensus
              210       220       230       240       250
191 S L A S L N D L S T E A R N H L D I A N Q L V A I D Q S I Q A C E R G S N S M L N L D Q Q Y E Q W  ScaE BR
185 S L P S L N D L S T E V R K L H L G I A N Q L V A I D Q G I Q V C E Q G S N S M L K L G - Q Y E Q L  ScaE GM
178 S L P S L N D L S T E V R K L H L G I A N Q L V A V D Q D I Q A C E R G S N S M L K L G - Q Y E Q L  ScaE IK
191 S L P S L N D L A T E V K K L H L G I G E Q L E D I D E S I K A C E Q G S N S M L K L G - Q Y E Q L  ScaE KP

    L D . L . . . S . . I . . I L Q . . . Q A . . A . . . . . . . . . . . . E . . Q . . . Q M Q . I . . M   Consensus
              260       270       280       290       300
241 L D N L Y S T S E E I V L I L Q T H V Q A G - A Q A I P - - - Q D E L N Q M H K Q M Q D I K K L M  ScaE BR
234 L D D L Y T I S E D I V Q I L Q N H A Q A D N A Q P I T E A R - D A E I N Q L Y Q Q M Q V I E K R M  ScaE GM
227 L D D L Y R T S E N I V Q I L Q N N V Q A G N A Q A I T P I I P Q D E L N Q M Y K Q M Q D I E T L M  ScaE IK
240 L D D L S T T S K N I L Q I L Q K H V Q A G N A P A T - - - - K N E L D Q M Y K Q M Q D I K T L M  ScaE KP

    P K F . Q D L . . L . . Q L . . I . . L I A . . D . R A . . . . . . . . . . . . . P G L S . . . I R   Consensus
              310       320       330       340       350
286 P K F Q D L S M L A T Q L R N I S N L I A T F D H R A - E I K P D E N - - G P V Q P G L S D N I R  ScaE BR
283 P K F T Q D L R I L A K Q L R D I S C L I A T L D P R A - K L D P A Q I G L Q Q V Q P G L S E K I R  ScaE GM
277 P K F T Q D L G M L A K Q L R D I S C L I A T L D P R A - E L D P A Q I G L Q Q V Q P G L S E K I R  ScaE IK
285 P K F T Q D L E M L S K Q L H D I L G L I A K L D P R A D E L D T T - - - - K H Q D P G L S E R I R  ScaE KP

    . . . . . . I I N D F P . . . L Q Q Y K N N L E N . L K . I N . K I A N I D Q L V S . L N Q . L . . L Q   Consensus
              360       370       380       390       400
333 - - - - E I I N D F P Q A L Q Q Y K N N L E N N L K S I N H K I A N I D Q L V S Y L N Q Q L D T L Q  ScaE BR
332 K I I N D I I N D F P Q V L Q Q Y K N N L E N D L K S I N H K I A N I D Q L V S S L N Q K L E A L Q  ScaE GM
326 K I V N D I I N D F P Q V L Q Q Y K N N L E N D L K S I N H K I A N I D Q L V S S L N Q K L D T L Q  ScaE IK
331 K I V N D I I N D F P P V L Q Q Y K N N L E N D L K I I N D K I A N I D Q L V S S L N Q K L D T L Q  ScaE KP
```

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 ≥1:12,800 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 detection 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 single gene, *tsa56*, encoding a 56-kDa major outer membrane protein, and on antibody responses against TSA56. Since *tsa56* is highly variable, the variation may affect PCR sensitivity (26), and more critically, multiple antigenic variants should be used to cover the diverse serotypes for serological detection of specific antibodies (10). Third, the method cannot differentiate between genetic variations of *O. tsutsugamushi* strains if restriction fragment length polymorphism (RFLP) mapping or sequencing is not performed. To date, more than 20 antigenically distinct strains have been reported (14). In addition to antigenic variation, multiple studies have shown great interstrain variability in virulence in humans and rodents (14). Therefore, differentiation of genetic variation is an important issue in scrub typhus diagnosis and epidemiology.

Here we report that Sca proteins of *O. tsutsugamushi* can induce antibody responses in scrub typhus patients. Specific antibody responses against ScaA and ScaC were observed mainly in scrub typhus patients with high IFA titers, so a serological test using the Sca antigens may not be efficient if used solely, especially during the early phase of infection. However, it is notable that ELISAs using ScaA or ScaC showed 100% sensitivity (Fig. 2 and Table 2), and the use of these antigens with TSA56 may synergistically enhance the sensitivity of serological diagnostic tests such as ICT. Previously, it was suggested that the 47-kDa protein, an HtrA homolog of *O. tsutsugamushi*, might be a useful antigen if combined with TSA56 for serological diagnosis (8, 15). However, specific antibody titers against the 47-kDa antigen in human patients

are relatively low during the acute phase of infection (8), which might be due to antibody cross-reaction with human serine protease or HtrAs of other bacteria (7). We also consistently observed relatively low levels of specific antibody responses against the 47-kDa antigen in our human patients (Fig. 3). Given that the amino acid sequences of Sca passenger domains are highly specific to *Orientia* and show low levels of sequence identity (<30%) with other rickettsial Sca proteins (12), these bacterial antigens may be effective targets for scrub typhus diagnosis. Although we may need to further confirm the antigenic variation of the diverse strains and the antibody responses against Sca antigens of different strains in human scrub typhus patients, the sequence conservation observed in this study supports the potential benefits of using Sca antigens as novel diagnostic targets in combination with TSA56.

In addition to their use in serological methods, the *sca* genes could also be useful targets for molecular diagnostic methods such as PCR-based detection. Currently, PCR-based 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.

ACKNOWLEDGMENT

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