

## Antigenic Drift of *Orientia tsutsugamushi* in South Korea as Identified by the Sequence Analysis of a 56-kDa Protein-Encoding Gene

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**Abstract.** This study was performed to determine the nationwide antigenic diversity of *Orientia tsutsugamushi* in South Korea. Sequence analysis was performed around variable domains I and II of a 56-kDa protein-encoding gene. We used eschar to overcome the disadvantages of conventional serotyping. The serological passive hemagglutination assay (PHA) was assessed based on the genotyping results. We analyzed 153 isolates from scrub typhus patients in major endemic areas and found that Boryong was the major strain (68.6%). New strains were also identified: Taguchi (19.6%), Kanda/Kawasaki (9.2%), and UAP7 (1.3%). PHA yielded significantly fewer positive results among Kawasaki strains ( $P < 0.001$ ), which are not included in the PHA antigen panel. In South Korea, Boryong was still the predominant strain, but the sequence analysis identified new changes in minor strains (30.1%). This antigenic drift had a negative effect on the PHA results. Periodic surveillance of the contemporary strains using sequence analysis is needed.

### INTRODUCTION

Scrub typhus, or tsutsugamushi disease, is an endemic disease in the Asia-Pacific region and shows various clinical manifestations. The causative organism, *Orientia tsutsugamushi*, enters the host through chigger bites on the skin, leaving a necrotic eschar lesion. The eschar is a good source of *O. tsutsugamushi* genetic material and is available for a relatively long time compared with the buffy coat, which is only useful early in the infection.<sup>1–3</sup> *O. tsutsugamushi* has over 20 antigenically distinct, regionally distributed serotypes.<sup>4</sup> Knowledge of serotype epidemiology is important for accurate serologic diagnosis and future vaccine development. Conventional serotyping of *O. tsutsugamushi* is limited to reference laboratories because of the complexity of the procedure and bio-safety considerations. This is one of the reasons that the prevalence data from endemic regions are sporadic<sup>4</sup> and that clinicians are not easily engaged in the clinical studies related to the serotypes or genotypes of *O. tsutsugamushi*. Since the first report of the nucleotide sequence of a 56-kDa protein-encoding gene of *O. tsutsugamushi* from the Karp strain, molecular methods have been used to determine variants of *O. tsutsugamushi* strains.<sup>5–7</sup> Although the correlation between the genotype and serological antigen type has not been entirely sorted out, gene-sequence analysis has become the major tool for genetic characterization of *Orientia* strains.<sup>4</sup> The genotyping method also has the advantage of detecting unknown serotypes that are not identified by assays that are based on reactions with monoclonal antibodies against known strains.

Scrub typhus is a communicable disease with a heavy disease burden in South Korea. Since the first confirmation of scrub typhus in native Koreans in 1985, the officially reported annual incidence of scrub typhus has increased markedly, with 6,057 cases reported in 2008 (<http://stat.cdc.go.kr>).<sup>8,9</sup>

We carried out this study to survey the prevalence of one time point cross-sectional genotypes of *O. tsutsugamushi* infecting humans throughout South Korea. Eschar was used as the source of *O. tsutsugamushi*. We analyzed whether genotypic variation had an effect on the performance of the diagnostic serological test widely used in clinical practices in South Korea.

### MATERIALS AND METHODS

**Study subjects.** The study was conducted prospectively from September to December 2006, when scrub typhus was at the peak of its seasonal outbreak. Patients were recruited from eight community-based hospitals (Sanggye Paik Hospital, Ilsan Paik Hospital, Pusan Paik Hospital, Dongguk University Ilsan Hospital, Dankook University Hospital, Namwon Medical Center, Chonbuk National University Hospital, and Sunlin Hospital) located at the major endemic areas of the country, with the exception of the northeastern area. We included clinically suspected scrub typhus patients over 16 years of age who had eschars and at least two of the following manifestations: fever, maculopapular skin rash, regional lymphadenopathy, headache, myalgia, cough, or abdominal discomfort. The eschar specimens were collected on either an inpatient or outpatient basis. The area where scrub typhus was acquired was estimated based on the clinical time sequence and the outdoor activity of the patient. The definite diagnosis of scrub typhus was based on the nucleotide sequence of a 56-kDa antigen gene of *O. tsutsugamushi* from each patient. Informed consent was obtained from the patients, and the study protocol was approved by the institutional review board at each participating hospital.

**Specimen collection and DNA extraction.** Most of the eschars were recovered during the dry stage to leave minimal damage to the normal skin tissue. Template DNA was extracted from eschars stored at  $-70^{\circ}\text{C}$  using a commercial kit according to the manufacturer's protocol (QIAamp DNA Mini Kit; QIAGEN, Valencia, CA). The template solution was recovered at a final volume of 200  $\mu\text{L}$ .

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**DNA amplification by polymerase chain reaction.** To genotype the *O. tsutsugamushi* strains, a gene fragment covering the variable domains (VDs) I and II of the 56-kDa antigen gene was amplified as previously described.<sup>10,11</sup> A set of primers, tsu-A (forward, 5'-TTT CGA ACG TGT CTT TAA GC-3'; nucleotide position -285 to -266 from the start codon of the 56-kDa gene based on the Gilliam strain) and tsu-B (reverse, 5'-ACA GAT GCA CTA TTA GGC AA-3'; 847-865), was used for the initial amplification. All polymerase chain reaction (PCR) reagents were used as recommended by the supplier (AccuPower PCR PreMix; Bioneer, Taejeon, South Korea). Briefly, 10  $\mu$ L of template DNA and 250  $\mu$ M of both primer tsu-A and primer tsu-B were used in each 50- $\mu$ L reaction mixture. PCR was initiated at 94°C for 2 minutes. Thermocycling was performed for 30 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1.5 minutes, and extension at 72°C for 2 minutes, with a final extension at 72°C for 7 minutes (GeneAmp PCR system 2400; Perkin-Elmer, Norwalk, CT). The final PCR products, estimated to be 1,151 bp in size, were confirmed by 2% agarose gel electrophoresis and then purified (QIAquick Gel Extraction Kit; QIAGEN, Valencia, CA).

**Sequence determination and phylogenetic analysis.** Direct sequencing of the PCR product was performed using three sequencing primers, tsu-E (forward, 5'-GTT GGA GGA ATG ATT ACT GG-3'; nucleotide position 124-143 from the start codon of the 56-kDa gene of the Gilliam strain), tsu-F (reverse, 5'-AGC GCT AGG TTT ATT AGC AT-3'; 731-749), and tsu-H (forward, 5'-GTT TAG AAT GGT TAC CAC-3'; -53 to -36).<sup>11,12</sup> The entire VDs I and II were expected to be included in the final sequence result. Sequencing reactions were performed on an MJ Research PTC-225 Peltier Thermo Cycler using an ABI PRISM BigDye Terminator Cycle Sequencing Kit with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA) following the protocols supplied by the manufacturer.

Initially, a nucleotide sequence with a maximum pairwise identity score with the 56-kDa antigen gene was identified from GenBank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for each sequence examined. The complete pool of reference sequences consisted of both the matching strains from GenBank and the general prototype strains. Then, both the study sequences and reference sequences were aligned using CLUSTAL W.<sup>13</sup> After the alignment, both the 5' and 3' ends of the aligned nucleotide sequences were trimmed to contain the entire VDs I and II of the 56-kDa gene and show even length endings.

Phylogenetic relationships among the study sequences and reference sequences were constructed by the neighbor-joining method and performed with 1,000 bootstrap replications to evaluate the reliability of the constructions.<sup>14,15</sup> A similarity matrix was constructed (Lasergene version 7.0, DNASTar, Madison, WI). The reference strains (accession number) from GenBank were Karp (M33004), Kato (M63382), Kawasaki (M63383), Kuroki (M63380), Shimokoshi (M63381), Boryong (AM494475), Yonchon (U19903), Sxh951 (AF050669), TA686 (U80635), TA678 (U19904), TA716 (U19905), TA763 (U80636), Ikeda (AF173033), Oishi (AF173037), Taguchi (AF173038), Kanda (AF173039), Omagari (AF173040), Matsuzawa (AF173043), Mori (AF173044), Okazaki (AF173045), Jecheon (AF430143), UAP7 (AF302995), and Hirahata (AF201835). The Gilliam (M33267) sequence was obtained from the literature.<sup>16</sup>

**Serological assay.** Baseline and follow-up blood samples were taken from patients at 7- to 14-day intervals. Paired sera

were used for serological tests by the passive hemagglutination assay (PHA). The PHA was performed with a commercial kit (Genedia Tsutsu PHA II, GreenCross SangA; Yongin city, Kyunggi, South Korea) designed for the quantitative and qualitative detection of antibodies against *O. tsutsugamushi* in human sera.<sup>17</sup> The test panel was comprised of antigens from the strains Karp, Gilliam, and Boryong, which are known to be prevalent in South Korea. A positive result was defined as a four-fold or greater change in the titer of the paired sera or a titer  $\geq 1:80$  in a single serum sample. The cut-off value of  $\geq 1:80$  was defined as a positive result by the manufacturer (Genedia Tsutsu PHA II, GreenCross SangA; Yongin city, Kyunggi, South Korea).

**Statistical analysis.** Pearson's  $\chi^2$  test was used for the categorical variables. A *P* value  $< 0.05$  was considered statistically significant.

## RESULTS

**Genotypes of *O. tsutsugamushi*.** One hundred ninety-two patients with eschar were enrolled from eight hospitals. Eschar specimens from all the patients were subjected to PCR. PCR was repeated several times for the specimens that yielded negative results to obtain the maximum number of positive results, and ultimately, 186 eschar specimens were PCR-positive. From these 186 PCR products, 153 sequences were appropriately determined and yielded nucleotide sequences corresponding to the 56-kDa antigen gene. According to the study design, these 153 patients were confirmed to have scrub typhus, and their data were used for the analysis.

The median age of the patients was 63 years (range = 18-91), and 90 patients (58.8%) were female; 55 patients (35.9%) had one or more underlying chronic diseases. These diseases included hypertension (21.6%), diabetes mellitus (8.5%), and other less frequent diseases such as congestive heart failure, cerebrovascular diseases with residual sequelae, chronic liver disease, and chronic obstructive lung disease requiring maintenance therapy. All patients survived 30 days.

The geographic distribution of the evaluated infections included seven provinces and covered most of the major endemic southern and western plain areas of South Korea (Chonbuk Province, 38.6%; Chungnam Province, 32.7%; Kyungnam Province, 9.8%; Chonnam Province, 2.6%) (Table 1). Northeastern and mideastern regions of the country are mostly mountainous, and cases from these areas were rare.

Genotypes matching the 153 recovered sequences were retrieved from GenBank. All of the sequences showed 99.6-100% homology with the corresponding reference sequences based on the 56-kDa antigen gene (Table 2). The length of the nucleotide sequences used for the comparison was 722 bp from the start codon of the 56-kDa antigen gene, using the Karp strain (M33004) as the reference for nucleotide position, and this sequence covered the entire VDs I and II. However, the study sequences were approximately 690-bp long, because they contained fewer nucleotide insertions than the reference sequences to which they were aligned. The majority of the isolates belonged to the Boryong strain (68.6%; 105 amplicons) and the Taguchi strain (19.6%; 30 amplicons). In addition, 14 sequences (9.2%) showed 100% homology for both the Kanda and Kawasaki strains. The Jecheon (1.3%; two amplicons) and UAP7 (1.3%; two amplicons) strains were the

TABLE 1  
Genotypic distribution of *O. tsutsugamushi* strains causing human infections in South Korea (N = 153)

Location (province)	Genotype					Total (%)
	Boryong	Taguchi	Kanda/Kawasaki	Jecheon	UAP7	
Kyunggi	4	1	–	1	2	8 (5.2)
Chungbuk	1	1	–	–	–	2 (1.3)
Chungnam	33	11	6	–	–	50 (32.7)
Chonbuk	51	5	2	1	–	59 (38.6)
Chonnam	2	2	–	–	–	4 (2.6)
Kyungbuk	3	9	3	–	–	15 (9.8)
Kyungnam	11	1	3	–	–	15 (9.8)
Total (%)	105 (68.6)	30 (19.6)	14 (9.2)	2 (1.3)	2 (1.3)	153 (100)

least common (Table 1). In regard to the reference strains, it was found that the Kuroki (M63380) and Boryong (AM494475) strains and the Kanda (AF173039) and Kawasaki (M63383) strains had identical sequences for the gene fragment analyzed in this study. These strains are antigenically different but could not be differentiated by our method.<sup>18,19</sup> Only the Boryong strain, and not the Kuroki strain, is mentioned in this study, because it is a long-term predominant strain and there has been no evidence of divergence. The term Kanda/Kawasaki is used for the 14 cases of Kanda or Kawasaki strains.

Phylogenetic relationships based on the alignment of VDs I and II of the 56-kDa antigen gene were determined to analyze the genetic divergence between the study sequences and reference sequences (Figure 1). The study sequences constituted two major clustered groups, the Boryong strain (SNU-I and -II) and Kawasaki strains (SNU-III and -V), with the remaining isolates being scattered. Although the cluster SNU-III was typed as a Kanda/Kawasaki strain and the cluster SNU-IV was typed as a Taguchi strain, both SNU-III and SNU-IV were called Kawasaki strains because of their close similarity (99.7–99.9%). The classic Kato strain was not found. The nucleotide sequences established in this study were deposited in GenBank under accession numbers EU523383–EU523531.

**Serological assay.** PHA results were available for 136 of the total 153 patients. Among the 95 patients infected with Boryong strains, 75 had a positive PHA result and 20 had a negative result compared with 9 positive and 31 negative PHA results among the 40 patients infected with Kawasaki strains (Kanda/Kawasaki + Taguchi). The PHA, thus, yielded significantly fewer positive results among Kawasaki strains, which are not included in the current PHA test panel ( $P < 0.001$ ) (Table 3).

DISCUSSION

Our study determined the nationwide *O. tsutsugamushi* genotypes causing human infections in South Korea. The antigenic diversity of *O. tsutsugamushi* has several implications. The serotypes of *O. tsutsugamushi* are related to vaccine development, clinical virulence, and appropriateness of the antigen panel used in the serological assay. Therefore, periodic surveillance of the circulating contemporary serotypes in actual human infections is important. However, the conventional surveillance approach has complex problems, including laborious procedures and biosafety, which need to be overcome to be readily accessible for investigators outside of the reference laboratory. To manage this problem, we chose genotyping based on the nucleotide sequence of the 56-kDa antigen gene and used clinical eschar as a source of *O. tsutsugamushi*. Furthermore, we expected that genotyping might further differentiate serotypes that cannot be further subclassified by the monoclonal antibody method.

The antigenic diversity of *O. tsutsugamushi* in South Korea has been reported in several studies. In a nationwide prevalence study in the early 1990s using the monoclonal antibody method, Chang<sup>20</sup> reported that 82.8% of 137 human cases were caused by the Boryong serotype and found less frequent cases caused by the Karp (13 cases; 9.7%) and Gilliam (10 cases; 7.5%) serotypes.<sup>20</sup> Several other published and unpublished data with small sample sizes show that the Boryong serotype is consistently predominant, but the other identified serotypes were rare and inconsistent.<sup>21</sup> Ree and others<sup>22</sup> characterized isolates ( $N = 41$ ) from mite pools from 1997 to 1999 using serotype-specific nested PCR.<sup>22</sup> The Boryong strain (82.9%) was predominant throughout the country, Karp strains (12.2%) were found in central South Korea, and the other strains were a mixture of both Boryong and Karp. No other strain was isolated. Shim and others<sup>23</sup> surveyed the genotypes isolated from wild rodents (60 isolates) and mite pools (129 isolates) in central and southern South Korea from 2005 to 2007 using the PCR-restriction fragment-length polymorphism method and sequence analysis of the 56-kDa antigen gene.<sup>23</sup> Boryong was the most common strain, with rates of 76.7% in rodents and 82.9% in mites. The other identified stains were Gilliam (10%), Pajoo (8.3%), and Karp (5%) in rodents and Karp (11.6%), Pajoo (3.1%), Yoncheon (1.6%), and Kawasaki (0.8%) in mites.

Our study is mostly an update for human infections and covers most of the endemic regions in South Korea. Our sequences contained the complete VDs I and II of the 56-kDa

TABLE 2  
Percent sequence similarity between study sequences and reference strains

	Study strains									Reference strains							
	S004	S010	S017	S048	S170	S189	S275	S284	S314	Boryong	Gilliam	Jecheon	Kanda	Karp	Kawasaki	Taguchi	UAP7
S004	–	91	92.1	82.2	91.3	82	91.4	82.2	91.3	91.4	83.4	99.9	82	96.3	82	82.2	92.1
S010		–	91.2	81.9	99.4	81.9	99.6	82	99.4	99.6	88.3	90.9	81.9	90.1	81.9	82	91.2
S017			–	82.5	91.2	82.4	91.4	82.5	91.2	91.4	83.6	92	82.4	90.8	82.4	82.5	100
S048				–	81.4	99.7	81.6	99.9	81.4	81.6	88.6	82	99.7	81.6	99.7	99.9	82.5
S170					–	81.3	99.9	81.4	99.7	99.9	87.7	91.1	81.3	90.1	81.3	81.4	91.2
S189						–	81.4	99.9	81.3	81.4	88.6	81.9	100	81.5	100	99.9	82.4
S275							–	81.6	99.9	100	87.8	91.3	81.4	90.3	81.4	81.6	91.4
S284								–	81.4	81.6	88.7	82	99.9	81.6	99.9	100	82.5
S314									–	99.9	87.7	91.1	81.3	90.1	81.3	81.4	91.2

Only representative sequences of clustered groups in Figure 1 were used for comparison, and clearly redundant identical sequences were not listed. S170 and S275 were from SNU-I, S314 was from SNU-II, S189 was from SNU-III, and S284 was from SNU-IV. S = SNU.

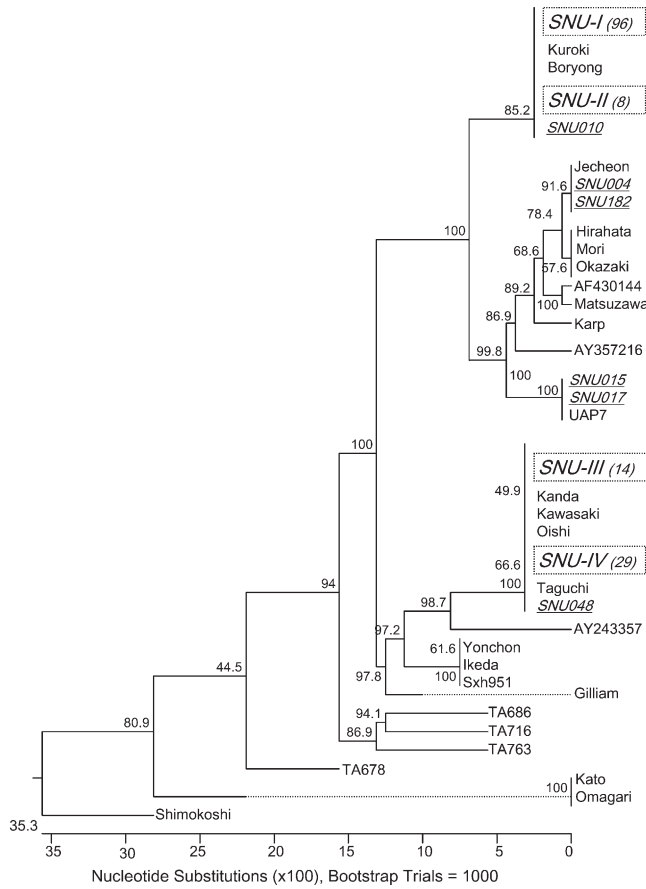


FIGURE 1. The phylogenetic tree was constructed by the neighbor-joining method based on VD I and II of the 56-kDa antigen gene of *O. tsutsugamushi* isolated from humans in South Korea. Large clusters of similar nucleotide sequences are grouped by dotted boxes and indicated as *SNU-I* to *-IV*. A basic local alignment search tool (BLAST) search confirmed the identification of *SNU-I* and *SNU-II* as Boryong strains, *SNU-III* as a Kanda/Kawasaki strain, and *SNU-IV* as a Taguchi strain. The numbers within parentheses indicate the number of isolates identified. Scattered isolates are indicated by individual underlined names such as *SNU\*\*\**. Reference strains were retrieved from GenBank. The numbers at the nodes indicate the percent reliability of each branch of the tree.

antigen gene, which shows the most variation in amino acid sequence. The Boryong strain was still dominant (68.6%), but previously unknown strains (Taguchi, Kanda/Kawasaki, and UAP7) were identified in 30.1% of the cases. Altogether, Boryong is undoubtedly the predominant strain in South Korea. However, there have been discrepancies regarding the

minor strains, and the typing methods are not comparable. Taguchi and Kanda/Kawasaki strains, which are clustered as Kawasaki strains, have not been previously reported in South Korea. These strains made up a relatively large portion of the human infections in our study, but they are not included in the antigen panel for the serological assays used in South Korea. We do not have a baseline for previous *O. tsutsugamushi* genotype data that is comparable with our results. This could be an indication of the changes in human cases that have taken place over the last two decades. Taguchi and UAP7 have never been isolated from rodents or mites in South Korea. Only one Kawasaki strain was isolated by another investigator during the same period as our study,<sup>23</sup> which may provide supporting evidence for the Kawasaki strains identified in our study.

Human infection by UAP7 has not been previously reported in the literature. This study is the first demonstration of UAP7 in a human infection since its initial isolation from rodents in Japan.<sup>12</sup> The two UAP7 cases were from northern South Korea and had severe clinical manifestations, including pneumonia. In a previous study, UAP7 reacted with an anti-Karp-specific monoclonal antibody and produced PCR products with a Karp type-specific primer pair.<sup>12</sup> Although we cannot extrapolate its frequency because of the limited number of UAP7 cases in our study, immunological methods would not distinguish between this strain and the Karp strain frequently isolated in northern South Korea.

Our specimens covered most of the endemic regions of South Korea. Although the number of specimens from the northern and northeastern regions was relatively small, the reported case incidence in those areas was also low. Kim HY (49th ICAAC poster presentation #B-031, unpublished data) characterized isolates ( $N = 40$ ) from 2006 to 2008 from the northeastern mountainous region of South Korea, which was not covered in our study, using nested PCR and sequence analysis of the final 487 bp of the 56-kDa antigen gene. That study identified 5 cases of the Boryong strain (12.5%), 2 cases of the Taguchi strain (5%), 22 cases of Karp-related strains (55%), 9 cases of Saitama strains (22.5%), and 2 cases of Japanese Karp (JP)-related strains (5%). In this region, the non-Boryong strains Gilliam and Karp were considered dominant.

Eschar is a necrotic tissue containing a mixture of *O. tsutsugamushi* and host inflammatory cells. Although the prevalence of eschars in scrub-typhus patients varies according to geographic region, they are identifiable in about 90% of patients from East Asian countries, including South Korea.<sup>24,25</sup> Genetic material from *O. tsutsugamushi* can be obtained from the tissue for more than 2 weeks, despite antibiotic treatment.<sup>1,26</sup> However, it is difficult to collect eschar during the wet, exudative early phase, and one sometimes obtains only thick exfoliative skin without any bacterial components. Positive results were obtained by PCR for 186 of the initial 192 eschar specimens, but for 33 of these instances, there was not enough PCR product to sequence. This was our first attempt to collect clinical eschars from multiple centers. With more experience and uniform techniques in specimen collection, the yield should be higher. In endemic regions with low eschar prevalence, the buffy coat would be an alternative source of *O. tsutsugamushi* genetic material, but this would only be successful during the early phase of infection or within 1 week of antibiotic therapy.<sup>1,26,27</sup>

The PHA method is widely used in South Korea because of its availability.<sup>28</sup> The indirect fluorescent antibody assay (IFA) has greater diagnostic sensitivity than PHA, but the overall

TABLE 3

The results of passive hemagglutination assay for 136 genotypes of *O. tsutsugamushi*

PHA results	Genotypes			
	Boryong	Taguchi	Kanda/Kawasaki	Jecheon
PHA-positive	75*	7	2	0
Single titer $\geq 1:80$	44	3	1	0
Follow-up titer rise $\geq$ four times	31	4	1	0
PHA-negative	20	22	9	1
Negative single titer	11	9	2	0
Negative follow-up titer rise	9	13	7	1
Total	95	29	11	1

\*  $P$  value  $< 0.001$  for Boryong strain vs. Kawasaki strains (Taguchi + Kanda/Kawasaki). PHA = passive hemagglutination assay.

diagnostic performance is also unsatisfactory.<sup>26</sup> Three antigens of Karp, Gilliam, and Boryong strains have been used in the test panels of both serological assays in South Korea. The present study showed that positive results from PHA were significantly lower with Kawasaki strains than with the Boryong strain; an antigen for the latter is included in the PHA test panel. This may explain why PHA has recently displayed lower sensitivity in clinical practice.<sup>26</sup> Considering our results, the best antigen panel for a serological assay to be used in South Korea would be comprised of Boryong, Karp, and Kawasaki antigens.

Our study was performed during a single outbreak season, and we only analyzed VDs I and II of the 56-kDa antigen gene. Previous data from the literature that we evaluated used different serotyping or genotyping methods from our study. These are limiting factors in the interpretation of our results. Therefore, periodic surveillance with uniform methods, preferably sequencing of the whole 56-kDa antigen gene, is needed to gain an accurate picture of the contemporary strains in the community.

In summary, we determined the circulating genotypes of *O. tsutsugamushi* strains causing human infections in South Korea by targeting the 56-kDa antigen gene and using clinical eschar specimens. This enabled us to identify previously unknown strains and determine the interval of change for the current circulating strains of *O. tsutsugamushi*. The serological assay lacked antigens that are relevant to the circulating strains and had significantly fewer positive results.

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