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Phylogenetic Analysis of the 56 kDa Protein Genes of *Orientia tsutsugamushi* in Southwest Area of Korea

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Abstract. This study was conducted to determine which genotypes were present in southwestern Korea. Nested polymerase chain reaction (PCR) and DNA sequence analysis targeting the *Orientia tsutsugamushi*-specific 56-kDa protein gene was performed with samples of blood and eschar. Of the 69 PCR-positive samples, 61 clustered with the Boryong previously isolated in Korea. CUH 4-6 had sequence homology of 100% with Kato, and CUH 4-3 had homology of 99.8% with Kato and formed the Kato cluster. CUH 4-57, CUH 4-31, CUH 4-142, and CUH 4-324 formed a Kawasaki cluster. CUH 4-271 had sequence homology of 100% with Jecheon and formed a Karp cluster. CUH 4-117 had homology of 99.8% with Neimeng-65, and Gilliam cluster. The most common genotype of *O. tsutsugamushi* in the southwestern part of Korea is the Boryong genotype. We also identified *O. tsutsugamushi* of the Kato, Neimeng-65 and Kawasaki genotypes, which had not been identified before in Korea.

INTRODUCTION

Scrub typhus is an acute febrile disease caused by *Orientia tsutsugamushi* (*O. tsutsugamushi*), which is widely distributed in rural areas of Southeast Asia.[1–3] *Orientia tsutsugamushi* characteristically has many variants, and eight scrub typhus antigen genes (Sta), namely Sta 150, 110, 72, 58, 56, 49, 47, and 20, have been identified.[4,5]

The 56-kDa type-specific antigen is a major outer membrane protein located on the surface of *Orientia* species that may be involved in penetration into host cells.[4] This 56-kDa antigen is an immunodominant antigen that induces strong humoral immunity. In addition, it contains both group-specific and type-specific epitopes, which are useful for the diagnosis of scrub typhus.[6] Ohashi and others[7] have demonstrated that the 56-kDa protein gene has variable domains that differ among strains. Three prototypes, the Karp, Kato, and Gilliam strains, account for the majority of isolates, and other serotypes such as Shimokoshi, Kuroki, Kawasaki, and Boryong, are also found because of variability of the main antigens.[8] Although the 56-kDa type-specific antigen differs between strains, its basic structure is constant and consists of ~1,600 base pairs.[4,7,9] It has been reported that the virulence of *O. tsutsugamushi* differs between strains depending on their serotype.[10] In the pre-antibiotic era, mortality was reported to be 45% in a certain region and 20% in another region.[11] This remarkable difference in mortality rate may be explained by regional strain differences, which implies that the virulence of *O. tsutsugamushi* is related to its serotype or genotype. Thus, it is important to investigate the regional distribution of serotypes and genotypes. Because each serotype of *O. tsutsugamushi* classified by differences in the antigen is associated with a specific variation of nucleotide sequences, further detailed genotypes may exist.

This study was conducted to determine which genotypes are present in the southwestern part of Korea by amplifying and sequencing the gene encoding the 56-KDa protein by means of the polymerase chain reaction (PCR).

MATERIALS AND METHODS

Patients. This study included adult patients ≥ 18 years of age with acute febrile disease who presented at our university hospital between September 1 and December 31, 2004, because of the presence of an eschar or maculopapular skin rash along with at least two of the following clinical symptoms: headache, generalized weakness, myalgia, cough, nausea, and abdominal discomfort. At that time, we collected and stored the samples of blood and eschar. In a previous study,[12] we performed PCR on eschars and blood buffy coats, together with indirect immunofluorescence assays (IFAs) on the sera, and we used the remaining specimens in this study. Scrub typhus was confirmed on the basis of either a single indirect immunofluorescent-specific immunoglobulin M (IgM) titer of ≥ 1:10 againt *O. tsutsugamushi* or a 4-fold or greater rise in the IFA IgG titer.[12]

Nested PCR. Because *Orientia* is an intracellular microorganism, the white blood cell buffy coat of whole blood was purified using a QIAamp DNA mini kit (Qiagen, Hilden, Germany).[12]

Nested PCR was performed by a modification of the method described by Furuya and others.[13] Nucleotide primers were based on the nucleotide sequence of the gene encoding the 56-kDa antigen of the Gilliam strain of *O. tsutsugamushi*.[13] Primers 34 (5-TCA AGC TTA TTG CTA GTG CAA TGT CTGC-3) and 55 (5-AGG GAT CCC TGC TGC TGT GCT TGC TGCG-3) were used in the first PCR. Nested PCR primers 10 (5-GAT CAA GCT TCC TCA GCC TAC TAT AAT GCC-3) and 11 (5-CTA GGG ATC CCG ACA GAT GCA CTA TTA GGC-3) were used in the second PCR amplification of the resulting 483 bp fragment (Figure 1). The primers amplified variable domains II and III of the 56-kD gene (Figure 1). All primers were purchased from Bioneer (Daejeon, Korea). The first round of PCR amplification was performed in a 20-μL reaction volume containing 2 μL of DNA, 1 μL of each 5 pmol/μL primer (forward and reverse primers), 10 μL of 2X EXCEL-Tag PreMix (Tag polymerase 2 U, 400 μm dNTP, 2.0 mM $MgCl_2$ KCL, Tris-Cl; Corebio, Seoul.,

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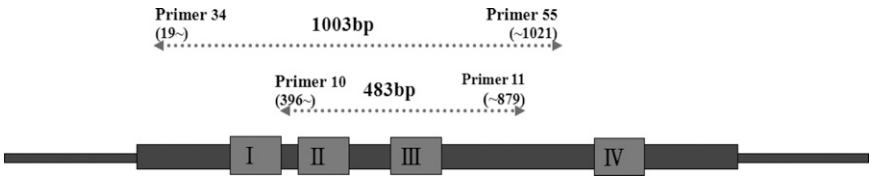

FIGURE 1. Positions of the primers used for 56-kDa gene amplification. The open reading frame of the gene is represented by the heavy line, and boxes I, II, III, and IV show the positions of the variable domains.

Korea) and sterile triple distilled water. The PCR reactions were carried out under the following conditions: initial denaturation at 94°C for 10 minutes, followed by 35 cycles, each consisting of denaturation at 94°C for 1 minute, 61°C for 1 minute and 72°C for 1 minute, and final extension at 72°C for 10 minutes. The second round of PCR amplification was conducted using 1 μL of the first PCR product as template DNA under the following conditions: initial denaturation at 94°C for 10 minutes, followed by 30 cycles, each consisting of denaturation at 94°C for 30 seconds, 63°C for 30 seconds and 72°C for 1 minute, and extension at 72°C for 7 minutes. The PCR was performed with a Biosystems Veriti 96-well thermal cycler (Applied Biosystems, Foster city, CA). The amplified PCR product was electrophoresed for 40 minutes on a 1.2% agarose gel (Seakem LE agarose) containing 0.5 ng/mL ethidium bromide at 100 volts (0.5X TAE, Bioneer) using a Bioneer electrophoresis machine.

Nucleotide sequences and phylogenetic analysis. The amplified PCR product was purified using a QIAquick gel extraction kit (Qiagen) and sent to Genotech (Daejeon, Korea) for sequencing with a 3730x1 DNA analyzer (Applied Biosystems, Foster, CA). The retrieved sequences were made with the basic local alignment search tool (BLAST) using database from the National Center for Biotechnology Information (NCBI) to determine the closest relatives, and newly identified nucleotide sequences were aligned with their closest relatives with the CLUSTAL X software program. The neighbor-joining method was used for phylogenetic analysis, and bootstrap was performed 1,000 times to increase phylogenetic reliability. The phylogenetic analysis was performed with the nucleotide sequences of the *O. tsutsugamushi* 56-kDa gene registered in Genbank that are distributed in many countries including Korea (Table 1).[7,14–20] The Tree Explorer was used to find the strain pedigree, and the LaserGene Program (DNASTAR, Madison, WI) was used to compare homologies. The clusters of genotypes in the strain pedigree were grouped by a modification of the method described by Tamura and others[15] and Fournier and others.[21]

RESULTS

The PCR was positive from the blood or eschar of 69 patients who were confirmed with scrub typhus. A phylogenetic tree was created of the 483 bps of 56-kDa genes of 67 *O. tsutsugamushi* strains registered in GenBank and their nucleotide sequences. With regard to nucleotide sequences, CUH 4-117 had the lowest similarity of 66.9% to LX-1 and TA686, whereas CUH4-271 was identical to the Jecheon strain, and CUH-4-6 was identical to the Kato and Omagari strains (Table 2). Sixty-one of the 69 genotypes found in this study, including CUH4-134 and CUH4-485, clustered together with strains related to the Boryong type. The 61 amplicons that belong to the Boryong cluster showed a minimum homology of 99.5% (CUH4-134) to the Boryong strain (accession no. AM 494475) and a maximum homology of 99.8% (CUH4-485) (Table 2). CUH4-6 was identical to the Kato and Omagari strains, and CUH4-3 had 99.8% homology to both the Omagari and Kato strains and clustered together with the Kato strains. The CUH4-31, CUH4-142, and CUH4-324 genotypes were 100% homologous to each other, and 99.8% homologous to both the Oishi and Taguchi strains and 99.5% each to Kawasaki and Kanda strains. CUH4-57 showed a homology of 99.8% to the Kawasaki and Kanda strains. CUH4-57, CUH4-31, CUH4-142, and CUH4-324 clustered together with the Kawasaki strains (Table 2, Figure 2). CUH4-271 had 100% homology to the Jecheon strain isolated in Korea and formed the Karp cluster together with the Hirahata, Kamimoto, and Matsuzawa strains. CUH4-117 had homology of 99.8% to the Neimeng-65 strain and clustered together with the Gilliam strain (Figure 2).

DISCUSSION

It has been reported that *O. tsutsugamushi* has a variety of serotypes that differ between endemic areas.[22–25] In Korea, the Boryong serotype was reported to be distributed throughout the country except for Cheju Island.[26] The Karp and Gilliam serotypes were prevalent in Taiwan, whereas the Gilliam serotype was prevalent in China.[27]

Thus, determining the serotypes in endemic areas is important for basic research on the classification of *O. tsutsugamushi*, the development of vaccines, and definitive diagnosis of scrub typhus. Expanding the panel of antigens used to test scrub typhus and to take into account local antigenic diversity would improve sensitivity of serologic diagnosis.[28] However, even though *O. tsutsugamushi* infection can be identified by serologic diagnosis, patient sera often cross-react with the antigens from different strains.[27] In our study, cross-reactions among Boryong, Gilliam, Karp, and Kato antigen were observed in most patients.

Genotypic identification using nested PCR and DNA sequencing methods could be useful.

The 16s rRNA gene of *O. tsutsugamushi* and its 56-kDa protein gene have been used for the diagnosis of scrub typhus. Because the 16s rRNA genes of the Gilliam, Karp, Kato, and Kuroki strains are ≥ 98.4% homologous,[29] this gene is useful for differentiating between *Orientia* and other genera/species, but not between *O. tsutsugamushi* strains. It has been reported that the 56-kDa protein gene is more useful for differentiating between *O. tsutsugamushi* strains than the 16s rRNA gene.[14]

TABLE 1
Strains of *Orientia tsutsugamushi* used in this study

Cluster*	Strain	Source (when available)	Geographical origin	GenBank accession no.	Reference
Karp	402I	Human	Japan	AF173047	14
Karp	Kamimoto	Human	Japan	AF173046	14
Karp	Mori	Human	Japan	AF173044	14
Karp	Okazaki	Human	Japan	AF173045	14
Karp	CMM1	Rodent	Japan	AF302986	15
Karp	KNP1	Rodent	Japan	AF302987	15
Karp	KNP2	Rodent	Japan	AF302988	15
Karp	Hirahata	Chiggers	Japan	AF173176	16
Karp	R39	Chiggers	Japan	AF201836	16
Karp	R9	Chiggers	Japan	AF201837	16
Karp	Jecheon	Human	Korea	AF430143	Unpublished
Karp	TW261	Rodent	Taiwan	AY222636	17
Karp	Karp	Human	New Guinea	AY956315	Direct Submission
Karp	Matsuzawa	Human	Japan	AF173043	14
Karp	Yeojoo	Human	Korea	AF430144	Unpublished
	TW73R	Rodent	Taiwan	AY222628	17
Saitama	TW121	Rodent	Taiwan	AY222639	17
Saitama	TW141	Rodent	Taiwan	AY222638	17
Saitama	TW441	Rodent	Taiwan	AY222634	17
Saitama	FAR1	Rodent	Japan	AF302989	15
Saitama	HSB1	Rodent	Japan	AF302983	15
Saitama	HSB2	Rodent	Japan	AF302984	15
Saitama	UAP1	Rodent	Japan	AF302991	15
Saitama	UAP2	Rodent	Japan	AF302992	15
Saitama	UAP4	Rodent	Japan	AF302993	15
Saitama	UAP7	Rodent	Japan	AF302995	15
Saitama	Pajoo	Human	Korea	AF430142	Direct Submission
Saitama	Yongworl	Human	Korea	AF430141	Direct Submission
	LA-1	Chiggers	Thailand	AF173049	14
	TW45R	Rodent	Taiwan	AY222632	17
	TW201	Rodent	Taiwan	AY222637	17
	TWyu81	Chiggers	Taiwan	AY222640	17
Boryong	Kuroki	Human	Japan	M63380	7
Boryong	Nishino	Human	Japan	AF173048	14
Boryong	Boryong	Human	Korea	AM494475	18
Kato	Kato	Human	Japan	M63382	7
Kato	Akita-7	Rodent	Japan	AF173041	14
Kato	Omagari	Rodent	Japan	AF173040	14
	LF-1	Chiggers	Thailand	AF173050	14
Kawasaki	Kawasaki	Human	Japan	M63383	7
Kawasaki	Kanda	Human	Japan	AF173039	14
Kawasaki	Oishi	Human	Japan	AF173037	14
Kawasaki	Taguchi	Human	Japan	AF173038	14
Gilliam	Gilliam		Taiwan	DQ485289	Unpublished
Gilliam	Neimeng-65		China	AF140143	Unpublished
Gilliam	TW461	Rodent	Taiwan	AY222631	17
Gilliam	405S	Human	Japan	AF173036	14
Gilliam	Ikeda	Human	Japan	AF173033	14
Gilliam	Iwataki-1	Rodent	Japan	AF173035	14
Gilliam	LP-1	Chiggers	Japan	AF173034	14
Gilliam	FAR2	Rodent	Japan	AF302990	15
Gilliam	HSB3	Rodent	Japan	AF302985	15
Gilliam	UAP6	Rodent	Japan	AF302994	15
Gilliam	Yonchon	Human	Korea	U19903	19
Gilliam	SXH951	Human	China	AF050669	Unpublished
	Shimokoshi	Human	Japan	M63381	7
	Fuji	Chiggers	Japan	AF201834	16
	LX-1	Chiggers	Japan	AF173042	14
	TA678	Rodent	Thailand	U19904	20
	TW381	Rodent	Taiwan	AY222635	17
	TW521	Rodent	Taiwan	AY222630	17
	TA763	Rodent	Thailand	U80636	20
	TA686	Rodent	Thailand	U80635	20
	TA716	Rodent	Thailand	U19905	20
	TW44R	Rodent	Taiwan	AY222633	17
	TW62R	Rodent	Taiwan	AY222629	17
	TWyu11	Chiggers	Taiwan	AY222641	17

* Cluster refers to the modified clustering system proposed by Tamura and others[15] and Fournier and others.[21]

TABLE 2

Homology of 56kDa gene nucleotide sequences among *Orientia tsutsugamushi* strains

	CUH 4-134	CUH 4-485	CUH 4-271	CUH 4-3	CUH 4-6	CUH 4-117	CUH 4-57	CUH 4-31	CUH 4-142	CUH 4-324
Hirahata	90.6	90.9	99.1	70.8	71	71.5	69.4	69.6	69.6	69.6
Mori	89.7	90	97.9	70.1	70.3	70.5	69.2	69.4	69.4	69.4
Kamimoto	89.7	90	97.9	70.1	70.3	70.5	69.2	69.4	69.4	69.4
Okazaki	89.7	90	97.9	70.1	70.3	70.5	69.2	69.4	69.4	69.4
Jecheon	90.6	90.9	**100**	70.3	70.5	71.2	69.2	69.4	69.4	69.4
Karp	91.1	91.3	99.1	70.5	70.8	71.2	69.9	70.1	70.1	70.1
Matsuzawa	89.5	89.7	96.8	70.8	71	70.8	69.9	70.1	70.1	70.1
Yeojoo	91.1	91.3	98.6	71	71.2	70.8	69.2	69.4	69.4	69.4
Yongworl	90.6	90.9	93.6	71	71.2	71.2	69.9	70.1	70.1	70.1
Pajoo	91.8	92	94.7	70.5	70.8	71.7	70.5	70.8	70.8	70.8
LA-1	90.6	90.9	93.6	70.8	71	72.1	71	71.2	71.2	71.2
Nishino	98.2	98.4	90.1	72.2	72.4	71	69.9	70.1	70.1	70.1
Boryong	**99.5**	**99.8**	91.3	71.7	72	71.7	70.6	70.8	70.8	70.8
Kuroki	**99.5**	**99.8**	91.3	71.7	72	71.7	70.6	70.8	70.8	70.8
Akita-7	73.2	73.5	72.3	99.5	99.8	75.6	75.6	75.4	75.4	75.4
Omagari	73.5	73.7	72.5	**99.8**	**100**	75.8	75.8	75.6	75.6	75.6
Kato	73.5	73.7	72.5	**99.8**	**100**	75.8	75.8	75.6	75.6	75.6
LF-1	73.9	74.2	73.2	94.6	94.8	78.9	77.2	77	77	77
Kanda	74.5	74.7	73.5	78.1	78.3	91.7	**99.8**	99.5	99.5	99.5
Kawasaki	74.5	74.7	73.5	78.1	78.3	91.7	**99.8**	99.5	99.5	99.5
Oishi	74.7	74.9	73.7	77.9	78.1	92	99.5	**99.8**	**99.8**	**99.8**
Taguchi	74.7	74.9	73.7	77.9	78.1	92	99.5	**99.8**	**99.8**	**99.8**
Neimeng-65	76.2	76.4	76.2	78.6	78.8	**99.8**	92.2	92.5	92.5	92.5
Gilliam	76.4	76.6	76.4	79.1	79.3	99.3	91.7	92	92	92
Ikeda	73.2	73.5	73.2	77.9	78.1	94.2	91.2	91	91	91
Iwataki-1	73.2	73.5	73.2	77.9	78.1	94.2	91.2	91	91	91
Yonchon	73.2	73.5	73.2	77.9	78.1	94.2	91.2	91	91	91
Shimokoshi	81	81.2	80.5	74.8	74.8	74.1	74.3	74.6	74.6	74.6
Fuji	74.5	74.7	72.1	70.2	70.4	70.2	71.2	70.9	70.9	70.9
LX-1	75.5	75.8	74.1	69.9	70.2	66.9	67.4	67.6	67.6	67.6
TA678	74.1	74.3	71.8	72.7	72.9	68.5	67.6	67.8	67.8	67.8
TA763	78.3	78.6	75.2	73.8	74	73.8	73.6	73.3	73.3	73.3
TA686	78.5	78.8	74.9	67.6	67.8	66.9	67.6	67.4	67.4	67.4
TA716	76.5	76.7	73.4	76.5	76.7	69.5	69.1	69.3	69.3	69.3

In this study, we amplified a 483 bp region containing VDII and VDIII of the *O. tsutsugamushi*-specific 56-kDa genes by nested PCR, because most genotypes can be differentiated at these loci because of the great variation.[30] The DNA sequences were compared with the nucleotide sequences of 67 *O. tsutsugamushi* registered in GenBank. The Boryong genotype was found to prevail in the southwestern part of Korea, which is consistent with previous reports, indicating that it is more prevalent in areas south to Choongnam Province of South Korea, whereas the Karp and Gilliam genotypes are prevalent in Kyounggi and Kangwon Provinces.[31,32] In the southwestern part of Korea, we identified CUH4-271 (Karp cluster), which had homology of 100% to the Jecheon genotype, and CUH4-3/CUH4-6 (Kato cluster), CUH4-31/CUH4-57/CUH4-142/CUH4-324 (Kawasaki cluster), and CUH4-117 (Gilliam cluster similar to Neimeng-65). However, further studies are needed to ascertain if there is a significant difference in the clinical manifestations and severity of scrub typhus according to genotype.

In conclusion, the results of this study confirmed that the Boryong genotype is most common in the southwestern part of Korea. In addition we identified Kato, Neimeng-65, and Kawasaki genotypes, which had not been encountered before in Korea. Thus, the results of this study confirm that various genotypes including the Boryong, Kato, Neimeng-65, Kawasaki, and Gilliam strains are present in this area.

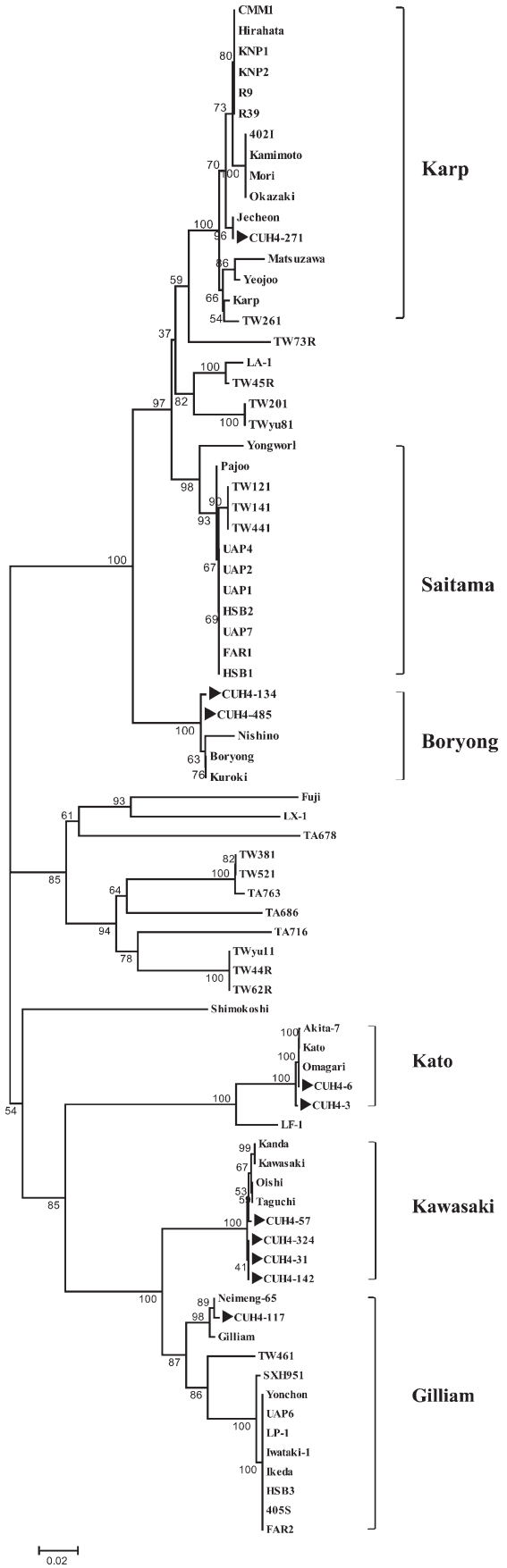

FIGURE 2. Phylogenetic tree based on the nucleotide sequences of *Orientia tsutsugamushi* 56-kDa genes.

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