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Communication

Isolation and Genetic Characterization of *Orientia tsutsugamushi* from Scrub Typhus Patients in Gyeongsangnam-do, Korea

Jong-Hun Ha[1], Dong-Hae Lee[1], Jin-Sik Park[1], Oh Hyun Cho[2], Dong-Hoon Kim[3], Na-Young Ha[4], Nam-Hyuck Cho[4], Kyu Jam Hwang[5], Yong-Gon Cho[6], Hye-Soo Lee[6], Won Kil Lee[7], Yoo Chul Lee[8], Myung-Je Cho[1], Woo-Kon Lee[1*] and Min-Kyoung Shin[1*]

Department of [1]Microbiology, [2]Internal Medicine, and [3]Emergency Medicine, Gyeongsang National University School of Medicine, Jinju; [4]Department of Microbiology and Immunology, Seoul National University College of Medicine, Seoul; [5]Pathogen Resource TF, Center for Infectious Diseases, Korea National Institute of Health, Korea Center for Disease Control and Prevention, Cheongju; [6]Department of Laboratory Medicine, Chonbuk National University Medical School, Jeonju; Department of [7]Laboratory Medicine, and [8]Microbiology, Kyungpook National University School of Medicine, Daegu, Korea

Orientia tsutsugamushi (*O. tsutsugamushi*), which is endemic to an Asia-Pacific region, has increased its incidence and caused annually around 10 thousand patients infected with scrub typhus in Korea in the past several years. In the present study, we isolated 44 *O. tsutsugamushi* from the patients with febrile illness accompanied with or without an eschar in Gyeongsangnam-do, Korea. These isolates were characterized by genetic analysis of the major outer membrane protein, the 56-kDa type-specific antigen (*tsa56*), which is unique to *O. tsutsugamushi*. Two types of sequences of *tsa56*, designated by JJ1 and JJ2, were determined from 37 and 7 isolates of the 44 isolates, respectively. JJ1 and JJ2 showed 74.7~90.8% identity in nucleotide sequence and 66.1~90.5% identity in amino acid sequence with 33 reference strains except for Boryong and Kuroki. JJ1 and JJ2 had 100 and 99.9% nucleotide identity to Boryong strain, and 99.9 and 99.8 % to Kuroki, which has been known to be similar to Boryong, respectively. In addition, they showed 77.9~ 81.4% nucleotide identity with the cluster of Gilliam-related genotypes, whereas they showed higher nucleotide identity (89.6~90.8%) with the cluster of Karp-related genotypes. To our knowledge, this is the first report to isolate *O. tsutsugamushi* and characterize their genotype as the Boryong in Jinju and West Gyeongsangnam-do, Korea, even though it has been reported that the Boryong was the predominant genotype in isolates from chiggers, domestic rodents, and patients in the southern part of Korea. Furthermore, our isolates could be useful source to study on the pathophysiology and epidemiology of scrub typhus in Korea.

Key Words: Scrub typhus, *Orientia tsutsugamushi*, 56-kDa type-specific antigen, *tsa56*, West Gyeongsangnam-do

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*Corresponding author: Woo-Kon Lee. Department of Microbiology, Gyeongsang National University School of Medicine, 816-15, Jinju-daero Jinju, Gyeong-Nam 52727, Korea.
Phone: +82-55-772-8083, Fax: +82-55-772-8089, e-mail: wklee@gnu.kr

*Corresponding author: Min-Kyoung Shin. Department of Microbiology, Gyeongsang National University School of Medicine, 816-15, Jinju-daero Jinju, Gyeong-Nam 52727, Korea.
Phone: +82-55-772-8081, Fax: +82-55-772-8089, e-mail: mkshin@gnu.kr

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INTRODUCTION

Scrub typhus is an acute febrile disease caused by infection with *Orientia tsutsugamushi* (*O. tsutsugamushi*), which is transmitted by a trombiculid mite (1). It is characterized by fever with eschar as a pathognomonic sign and accompanied by other non-specific clinical signs such as headache, chills, cough, myalgia, conjunctival injection and skin rash, and sometimes serious complications such as meningitis, disseminated intravascular coagulation and multi organ failure (2, 3). The prevention of scrub typhus via mechanical control such as avoiding mite bites is the best because there is no available vaccine, and infection may be fatal without aggressive medical treatment (4). Genotypes of *O. tsutsugamushi* strains were classified according to the diversity of outer membrane protein 56-kDa type-specific antigen (*tsa56*): Gilliam, Karp, Kato, Kawasaki, Kuroki, Shimokoshi, Boryong, Yonchon, and so on (3). The *tsa56*, which is essential to attach and penetrate to cells, has four variable domains I - IV and they show genotypes specificity (5, 6). Virulence of *O. tsutsugamushi* for humans varies depending on its genotypes. Therefore, the surveillance of geographical distribution of *O. tsutsugamushi* genotypes has been continuously performed.

O. tsutsugamushi is endemic to the geographical triangle, called as a tsutsugamushi triangle, of an Asia-Pacific region covering from northern Japan in the east to Pakistan in the west and northern Australia in the south (3, 7). The distribution of genotypes varies geographically. The Gilliam, Karp, Kato, TA678, Ta686, TA716, TA763, and TH1817 have been identified in Thailand, Malaysia, Philippines, and Australia (6). The Gilliam, Karp, Kato, Kawasaki, Shimokoshi, Irie, and Hirano were identified in Japan (6). TA716 and Gilliam were predominant in Australia and Russia, respectively (8). Meanwhile, the Karp and TA716 were predominant in Taiwan (8). The Boryong was the most common in southern part of Korea, but Karp and Gilliam were identified in northern and central part of Korea (6). *O. tsutsugamushi* isolates from chigger mites, field rodents, and human were identified as the Boryong in Gyeongsangnam-do, the southern part of Korea according to the reports of Chang *et al*. and Ree *et al*. in 1999 and 2001 (6, 9). Even though the Boryong shows 99.9% homology of the nucleotide and amino acid sequences of *tsa56* variable domain in comparison with the Kuroki, it has different features from the Kuroki in terms of virulence in mice and reactivity to KP10, a Karp-specific monoclonal antibody (2). In the present study, we isolated 44 *O. tsutsugamushi* from the patients with scrub typhus in Jinju, Korea and characterized their genotypes using tsa56.

MATERIALS & METHODS

Isolation and identification of *O. tsutsugamushi* from the patients with scrub typhus

O. tsutsugamushi isolates used in this study were obtained from National Culture Collection for Pathogens (NCCP, Cheongju, Korea). *O. tsutsugamushi* isolates were isolated and identified from the patients enrolled in Gyeongsang National University Hospital, Gyeongsangnam-do, Korea in 2015 as follows: Blood samples were drawn from the patients who had high fever and an eschar in 2015. Buffy coat was collected after centrifugation at 3,000 rpm for ten min and inoculated into L-929 (mouse fibroblast) cell culture. The inoculated cells were incubated in the presence of 5% CO_2 at 37℃ for one half-hour, and then washed with Dulbecco's phosphate buffered saline (DBPS; Gibco BRL Co., Gaithersburg, USA) after removal of the supernatant. The infected cells were maintained with media change twice a week under conditions of 5% CO_2 at 35℃ until the cytopathic effect appeared. Orientia infection was identified using indirect immunofluorescence assays (IFA) and PCR and sequencing for *tsa56*, as previously described (10, 11). For IFA, the infected cell culture prepared on slide glass were diagnosed using pooled serum from ten patients with scrub typhus whose IFA titers were more than 1:128. After *O. tsutsugamushi* was identified by IFA, the cells were detached using a scraper, and stored at - 80℃. All the isolates identified were then deposited at NCCP.

Amplification and sequencing of the *tsa56*

Amplification reactions of *tsa56* were performed using genomic DNA, which was extracted from the infected L-929

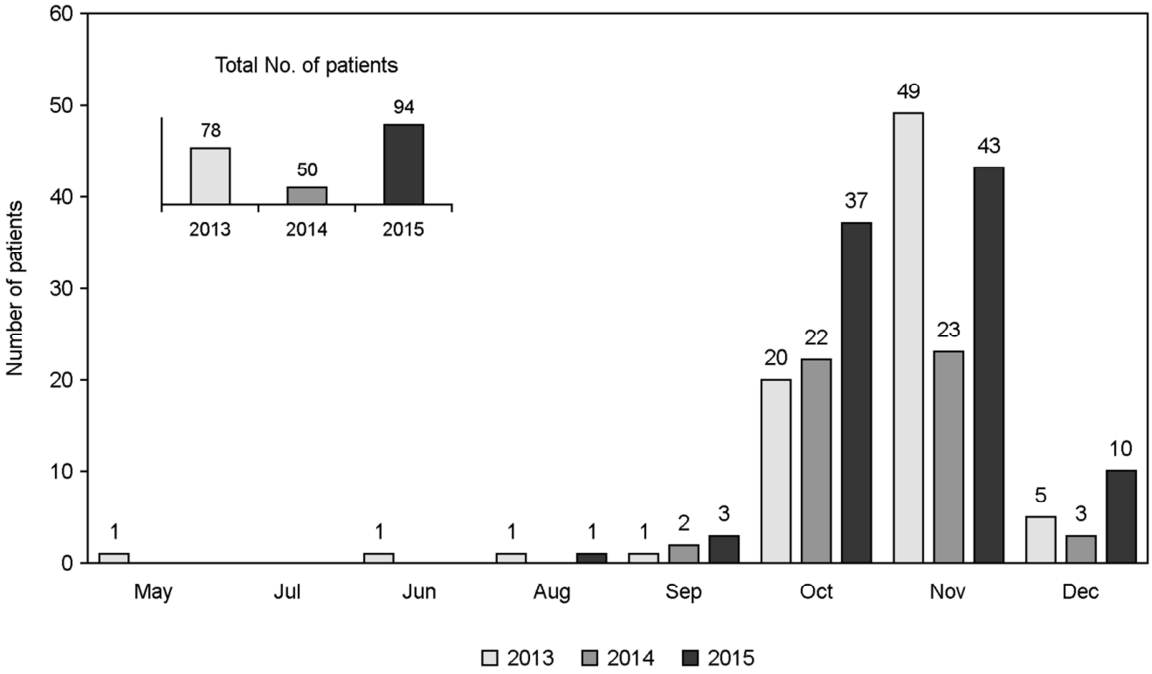

Figure 1. The incidence of the scrub typhus patients of Gyeongsang National University Hospital in 2013 to 2015.

cell using DNeasy tissue kits (QIAGEN, Hilden, Germany) according to manufacturer's protocol. The primer set for *tsa56* gene are p33 (forward): 5'-TCAAGCTTATTGCTA-GTGCAATGTCTGC-3', and p55 (reverse): 5'-AGGGAT-CCCTGCTGCTGTGCTTGCTGCG-3' according to the method of Furuya *et al*. (11). The PCR was performed as the following conditions: an initial denaturation step at 94℃ for 7 min was followed by 35 cycles of 94℃ for 1 min, 57℃ for 1 min, and 72℃ for 1 min, and then a final incubation step of 72℃ for 10 min. The PCR products were purified using the GeneAll PCR SV (GeneAll, Seoul, Korea) and sequenced using automatic dye terminator DNA sequencing (Thermo Fisher Scientific, Waltham, USA).

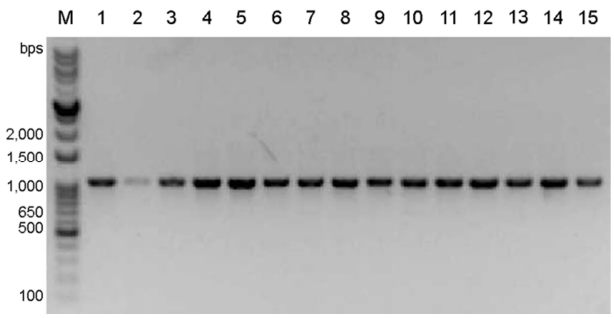

Figure 2. The PCR products of the 56-kDa type-specific antigen gene of *O. tsutsugamushi* from 15 representative isolates in the present study. Forty-four *O. tsutsugamushi* isolates were confirmed by *tsa56*-specific PCR assay, and PCR products of 15 representative isolates among 44 isolates were presented. M, DNA sizer marker.

Phylogenetic analysis

The sequence data were aligned with reference sequences of *tsa56* in the BLASTN database (National Center for Biotechnology Information) using MegAlign 5.0 DNASTAR (DNASTAR Inc., Madison, USA). Nucleotide identity of the gene was calculated on the basis of pairwise comparison using Lasergene, 5.0 DNASTAR. Phylogenetic relationships were generated via Neighbor-joining methods, bootstrap analysis (500 repeats) using MEGA software (version 6.0, Arizona State University, Tempe, USA).

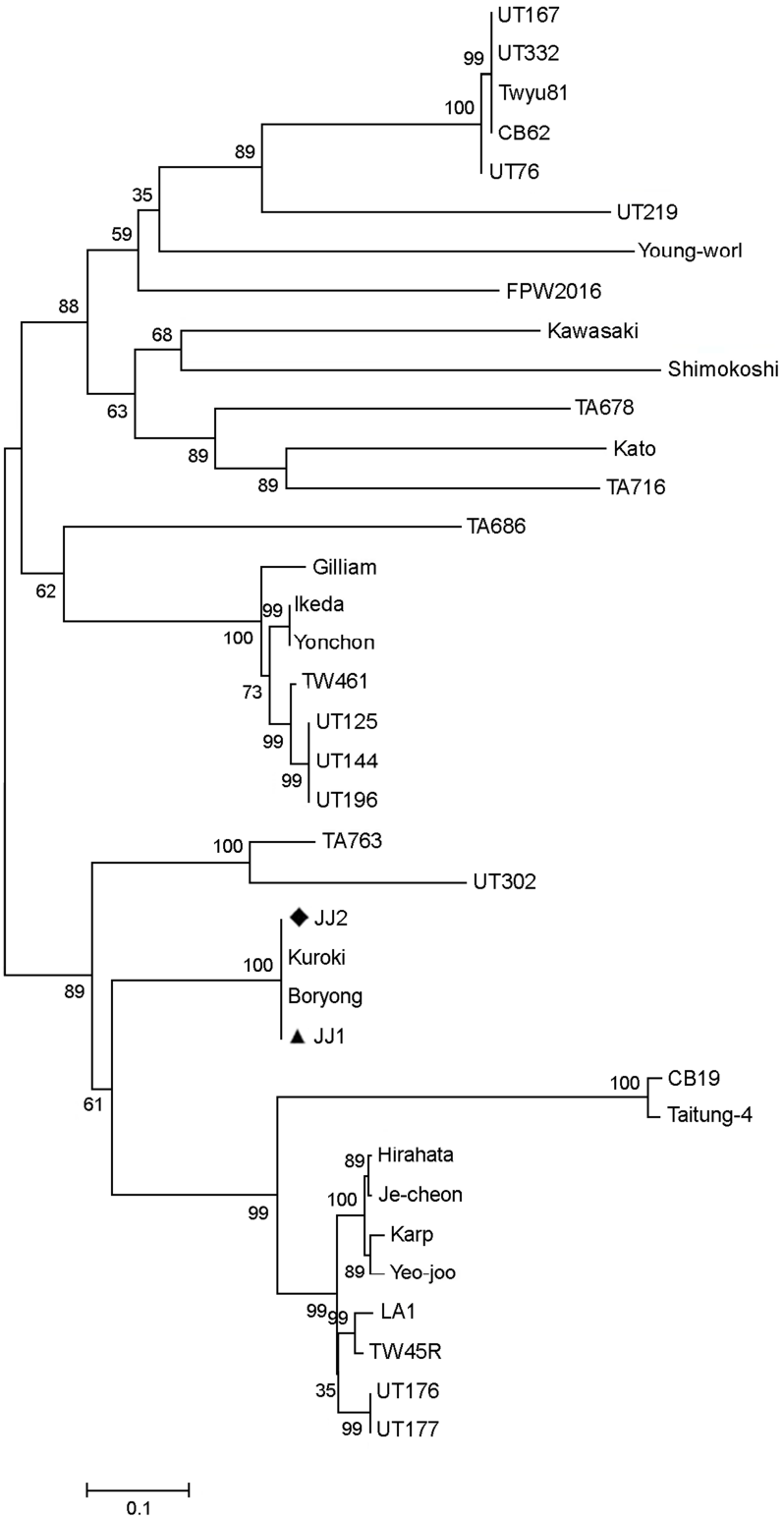

Figure 3. Phylogenetic tree based on partial 56-kDa type specific antigen of *O. tsutsugamushi*. Two nucleotide sequences of *O. tsutsugamushi* isolates were analyzed phylogenetic relationship with reference sequences retrieved from the GenBank database as shown in Table 1. Solid triangle and diamond indicate two sequences of *O. tsutsugamushi* determined in this study.

Table 1. Sequence homologies of partial 56-kDa type specific antigen gene between *Orientia tsutsugamushi* strains

Isolates	Nucleotide identity (%)		Amino acid identity (%)		Information				
	JJ1	JJ2	JJ1	JJ2	Source	Country	Year	Accession no.	Reference
Boryong	100	99.9	100	100	Human	South Korea	1998	L04956	(9)
CB19	77.9	78.1	68.1	68.1	*Rattus rattus*	Thailand	2009	GU068058	(13)
CB62	85.8	85.6	78.3	78.3	*Bandicota indica*	Thailand	2009	GU068055	(13)
FPW2016	78.1	77.9	67	67	Human	Thailand	2007	EF213085	(14)
Gilliam*	86.5	86.4	78.3	78.3	Human	Assam-Burma border	1943	DQ485289	(15)
Hirahata	90.8	90.7	90.5	90.5	*Leptotrombidium pallidum*	Taiwan	1999	AF201835	(16)
Ikeda	81.2	81.1	72.3	72.3	Human	Japan	1979	AF173033	(17)
Je-cheon	90.7	90.6	84.2	84.2	Human	South Korea	2001	AF430143	–
Karp*	90.6	90.5	84.6	84.6	Human	New Guinea	1943	AY956315	(18)
Kato*	76.9	76.8	66.1	66.1	Human	Japan	1955	AY836148	(19)
Kawasaki	78.0	77.9	66.5	66.5	Human	Japan	1981	M63383	(20)
Kuroki	99.9	99.8	100	100	Human	Japan	1981	M63380	(21)
LA-1	90.0	89.9	82.8	82.8	*Leptotrombidium arenicola*	Malaysia	1993	AF173049	(22)
LF-1	76.2	76.1	66.8	66.8	*Leptotrombidium fletcheri*	Malaysia	1993	AF173050	(22)
Shimokoshi	74.8	74.7	61.4	61.4	Human	Japan	1980	M63381	(17)
TA678	78.1	78.2	67	67	*Rattus rattus*	Thailand	1963	U19904	(23)
TA686	82.0	82.1	73.1	73.1	*Tupaia glis*	Thailand	1963	U80635	(23)
TA716	78.3	78.4	67.8	67.8	*Menetes berdmorei*	Thailand	1963	U19905	(23)
TA763	84.1	84.2	74.6	74.6	*Rattus rajah*	Taiwan	1963	U80636	(23)
Taitung-4	78.9	79.0	70.2	70.2	–	Taiwan	2004	AY787232	–
TW45R	90.7	90.6	84.2	84.2	*Rattus losea*	Taiwan	2003	AY222632	(24)
TW461	81.1	81.0	71	71	*Rattus rattus*	Taiwan	2003	AY222631	(24)
TWyu81	86.1	86.0	77.9	77.9	*Leptotrombidium pallidum*	Thailand	2003	AY222640	(24)
UT76	85.9	85.8	71	71	Human	Thailand	2007	EF213078	(14)
UT125	81.4	81.2	71	71	Human	Thailand	2007	EF213096	(14)
UT144	81.4	81.2	78.3	78.3	Human	Thailand	2007	EF213091	(14)
UT167	86.1	86.0	80.6	80.6	Human	Thailand	2007	EF213080	(14)
UT176	89.8	89.6	80.6	80.6	Human	Thailand	2007	EF213081	(14)
UT177	89.8	89.6	71	71	Human	Thailand	2007	EF213084	(14)
UT196	81.4	81.2	86.3	86.3	Human	Thailand	2007	EF213079	(14)
UT219	90.6	90.5	69.5	69.5	Human	Thailand	2007	EF213100	(14)
UT302	79.4	79.6	78.3	78.3	Human	Thailand	2007	EF213095	(14)

Table 1. Sequence homologies of partial 56-kDa type specific antigen gene between *Orientia tsutsugamushi* strains (Continued)

Isolates	Nucleotide identity (%)		Amino acid identity (%)		Information				
	JJ1	JJ2	JJ1	JJ2	Source	Country	Year	Accession no.	Reference
UT332	86.1	86.0	77.9	77.9	Human	Thailand	2007	EF213083	(14)
Yeojoo	90.4	90.2	84.2	84.2	Human	South Korea	2001	AF430144	–
Yonchon	81.2	81.1	72.1	72.1	Human	South Korea	1989	U19903	(25)
Young-worl	87.8	87.7	79.4	79.4	Human	South Korea	2001	AF430141	–

*Original prototype strains

RESULTS & DISCUSSION

As shown in Fig. 1, the total number of the scrub typhus patients diagnosed in Gyeongsang National University Hospital increased since 2013, even though the number of patients decreased somewhat in 2014. The incidence was focused on from October to December, and was the highest in November. Ninety-four cases were diagnosed or suspected as incidence of the scrub typhus from the patients with acute febrile illness with or without eschar in 2015. Among the ninety-four cases, blood samples were collected from 57 patients and 44 isolates of *O. tsutsugamushi* were finally obtained and deposited at NCCP.

Forty-four isolates from Gyeongsangnam-do in 2015 were obtained from NCCP, and the identification was confirmed by PCR amplification of *tsa56*, which was predicted to be a length of 1,028-bp (Fig. 2). A total 44 isolates were obtained from 26 women and 18 men. In addition, age distribution of the scrub typhus patients was 10~80 years. The number of patients for each age group is as follows: 10s, 1; 30s, 1; 40s, 1; 50s, 10; 60s, 6; 70s, 20; and 80s, 5.

In the present study, nucleotide sequence of *tsa56* from 44 isolates, which is a species-specific marker gene of *O. tsutsugamushi* was divided into two types, JJ1 and JJ2. JJ1 and JJ2 were determined from 37 and 7 isolates, respectively. Therefore these two sequences and 35 reference sequences of *tsa56* were enrolled in the nucleotide and amino acid identity analysis and phylogenetic analysis. JJ1 and JJ2 showed 74.7~90.8% nucleotide identity and 66.1~90.5% amino acid identity with 33 reference sequences except for Boryong and Kuroki. Since both Boryong and Kuroki strains showed 100% and 99.9% nucleotide identities with JJ1 and 99.9% and 99.8% to JJ2, they were clustered within the same phylogenic tree (Table 1 and Fig. 3). In addition, they showed 77.9~81.4% nucleotide identity with the cluster of Gilliam-related genotypes consisting of FPW2016, Gilliam, Ikeda, Kawasaki, TW461, UT125, UT144, UT196, and Yonchon, whereas they showed higher nucleotide identity (89.6~90.8%) with the cluster of Karp-related genotypes consisting of Hirahata, Je-cheon, Karp, LA-1, TW45R, UT176, UT177, Yeojoo (Table 1 and Fig. 3).

In the previous report, Chong *et al*. isolated 11 strains of *O. tsutsugamushi* from scrub typhus patients in Jinhae, which is central area of Gyeongsangnam-do and identified 10 of them as the Karp type based on the indirect immunoperoxidase test with monoclonal antibodies specific to Gilliam, Karp or Kato strain (12). Even though they were reported as Karp type in 1989, they could be considered the Boryong type since the Boryong was determined as a new type in 1990. Moreover, most *O. tsutsugamushi* isolates from chigger and rodents in Gyeongsangnam-do have identified as the Boryong. The fact that the amino acid identities of JJ1 and JJ2 are the highest in the comparison with the Karp type (84.6%) among the three original prototypes (Gilliam, Karp and Kato) in this study (Table 1) can partially explain the reason why they were identified as the Karp type serologically in the previous report.

To our knowledge, this is the first report to isolate *O. tsutsugamushi* characterized as their genotypes to the Boryong

in Jinju and nearby area, Gyeongsangnam-do, Korea, even though it has been reported that the Boryong was the predominant genotype isolated from chiggers, domestic rodents, and patients in the southern part of Korea. Our isolates could be useful source for microbiological and epidemiological study on *O. tsutsugamushi* infection in Korea.

REFERENCES

1) Tamura A, Ohashi N, Urakami H, Miyamura S. Classification of *Rickettsia tsutsugamushi* in a new genus, Orientia gen. nov., as *Orientia tsutsugamushi* comb. nov. Int J Syst Bacteriol 1995;45:589-91.
2) Kim DM, Yun NR, Neupane GP, Shin SH, Ryu SY, Yoon HJ, *et al*. Differences in clinical features according to Boryoung and Karp genotypes of *Orientia tsutsugamushi*. PLoS One 2011;6:e22731.
3) Yang HH, Huang IT, Lin CH, Chen TY, Chen LK. New genotypes of *Orientia tsutsugamushi* isolated from humans in Eastern Taiwan. PLoS One 2012;7:e46997.
4) Nakayama K, Yamashita A, Kurokawa K, Morimoto T, Ogawa M, Fukuhara M, *et al*. The Whole-genome sequencing of the obligate intracellular bacterium *Orientia tsutsugamushi* revealed massive gene amplification during reductive genome evolution. DNA Res 2008;15: 185-99.
5) Shirai A, Tanskul PL, Andre RG, Dohany AL, Huxsoll DL. *Rickettsia tsutsugamushi* strains found in chiggers collected in Thailand. Southeast Asian J Trop Med Public Health 1981;12:1-6.
6) Ree HI, Kim TE, Lee IY, Jeon SH, Hwang UW, Chang WH. Determination and geographical distribution of *Orientia tsutsugamushi* serotypes in Korea by nested polymerase chain reaction. Am J Trop Med Hyg 2001; 65:528-34.
7) Zhang M, Zhao ZT, Yang HL, Zhang AH, Xu XQ, Meng XP, *et al*. Molecular epidemiology of *Orientia tsutsugamushi* in chiggers and ticks from domestic rodents in Shandong, northern China. Parasit Vectors 2013;6:312.
8) Kelly DJ, Fuerst PA, Ching WM, Richards AL. Scrub typhus: the geographic distribution of phenotypic and genotypic variants of *Orientia tsutsugamushi*. Clin Infect Dis 2009;48 Suppl 3:S203-30.
9) Chang WH, Kang JS, Lee WK, Choi MS, Lee JH. Serological classification by monoclonal antibodies of *Rickettsia tsutsugamushi* isolated in Korea. J Clin Microbiol 1990;28:685-8.
10) Yamashita T, Kasuya S, Noda N, Nagano I, Kang JS. Transmission of *Rickettsia tsutsugamushi* strains among humans, wild rodents, and trombiculid mites in an area of Japan in which tsutsugamushi disease is newly endemic. J Clin Microbiol 1994;32:2780-5.
11) Furuya Y, Yoshida Y, Katayama T, Yamamoto S, Kawamura A, Jr. Serotype-specific amplification of *Rickettsia tsutsugamushi* DNA by nested polymerase chain reaction. J Clin Microbiol 1993;31:1637-40.
12) Chong YS, Kim JJ, Kwak CG, Lee SY, Yi KS, Suto T. Isolation of *Rickettsia tsutsugamushi* from patients in Chinhae area in 1987. J Korean Soc Microbiol 1989;24: 81-7.
13) Rodkvamtook W, Ruang-Areerate T, Gaywee J, Richards AL, Jeamwattanalert P, Bodhidatta D, *et al*. Isolation and characterization of *Orientia tsutsugamushi* from rodents captured following a scrub typhus outbreak at a military training base, Bothong district, Chonburi province, central Thailand. Am J Trop Med Hyg 2011;84: 599-607.
14) Blacksell SD, Luksameetanasan R, Kalambaheti T, Aukkanit N, Paris DH, McGready R, *et al*. Genetic typing of the 56-kDa type-specific antigen gene of contemporary *Orientia tsutsugamushi* isolates causing human scrub typhus at two sites in north-eastern and western Thailand. FEMS Immunol Med Microbiol 2008; 52:335-42.
15) Bengston IA. A serological study of 37 cases of tsutsugamushi disease (scrub typhus) occurring in Burma and the Philippine Islands. Public Health Rep 1946;61:887 -94.
16) Tamura A, Makisaka Y, Kadosaka T, Enatsu T, Okubo K, Koyama S, *et al*. Isolation of *Orientia tsutsugamushi* from Leptotrombidium fuji and its characterization. Microbiol Immunol 2000;44:201-4.
17) Tamura A, Takahashi K, Tsuruhara T, Urakami H, Miyamura S, Sekikawa H, *et al*. Isolation of *Rickettsia tsutsugamushi* antigenically different from Kato, Karp, and Gilliam strains from patients. Microbiol Immunol 1984;28:873-82.

18) Derrick EH, Brown HE. Isolation of the Karp strain of rickettsia tsutsugamushi. Lancet 1949;2:150.
19) Shishido A, Ohtawara M, Hikita M, Kitaoka M. The nature of immunity against scrub typhus in mice. II. The cross-protection test with mice for identification and differentiation of several strains of *Rickettsia orientalis* newly isolated in Japan. Jpn J Med Sci Biol 1959;12: 391-404.
20) Yamamoto S, Kawabata N, Tamura A, Urakami H, Ohashi N, Murata M, *et al*. Immunological properties of *Rickettsia tsutsugamushi*, Kawasaki strain, isolated from a patient in Kyushu. Microbiol Immunol 1986;30: 611-20.
21) Ohashi N, Tamura A, Sakurai H, Yamamoto S. Cha-acterization of a new antigenic type, Kuroki, of *Rickettsia tsutsugamushi* isolated from a patient in Japan. J Clin Microbiol 1990;28:2111-3.
22) Enatsu T, Urakami H, Tamura A. Phylogenetic analysis of *Orientia tsutsugamushi* strains based on the sequence homologies of 56-kDa type-specific antigen genes. FEMS Microbiol Lett 1999;180:163-9.
23) Shirai A, Robinson DM, Brown GW, Gan E, Huxsoll DL. Antigenic analysis by direct immunofluorescence of 114 isolates of *Rickettsia tsutsugamushi* recovered from febrile patients in rural Malaysia. Jpn J Med Sci Biol 1979;32:337-44.
24) Qiang Y, Tamura A, Urakami H, Makisaka Y, Koyama S, Fukuhara M, *et al*. Phylogenetic characterization of *Orientia tsutsugamushi* isolated in Taiwan according to the sequence homologies of 56-kDa type-specific antigen genes. Microbiol Immunol 2003;47:577-83.
25) Seong SY, Park SG, Huh MS, Jang WJ, Choi MS, Chang WH, *et al*. T-track PCR fingerprinting for the rapid detection of genetic polymorphism. FEMS Microbiol Lett 1997;152:37-44.