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Phylogenetic Clustering of 4 Prevalent Virulence Genes in *Orientia tsutsugamushi* Isolates from Human Patients

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The pathogenicity of microbes is involved in many kinds of virulence genes. The relationships between these virulence genes and strains are not clear in *Orientia tsutsugamushi* yet. In this study, we confirmed the presence of the virulence genes and classified into *O. tsutsugamushi* isolates using phylogenetic analysis of the virulence genes. We also compared the fatality rates of every isolate via an infection experiment in BALB/c mice using the *O. tsutsugamushi* isolates, Deajeon03-01, Wonju03-01, and Muju03-01. Moreover, we compared the phylogenetic analysis, in basis with 56 kDa protein sequence which determined from serotype, and virulence genes of *O. tsutsugamushi*. Our results showed remarkably different fatality rates between Deajeon03-01 and Muju03-01, which are both Boryong strains of *O. tsutsugamushi*. Also, clustering analyses including these two isolates gave slightly different results depending on whether they were clustered based on virulence genes or on the 56 kDa protein sequences. Consequently, we conclude that fatality rates in *O. tsutsugamushi* are correlated with differences in both serotypes and virulence genes. We identified some variations within the virulence genes *dnaA*, *virB8*, *tolR*, and *trxA* among the isolates.

***Keywords*:** *O. tsutsugamushi*, virulence gene, phylogenetic analysis

Orientia tsutsugamushi, a Gram-negative, obligate intracellular bacterium, is the etiological agent of tsutsugamushi desease (scrub typhus) (Philip, 1948; Ohashi *et al*., 1995). It is maternally inherited in *leptotrombidium* mites and can be transmitted to humans by feeding larvae (Traub and Wisseman, 1974). Tsutsugamushi disease is characterized by fever, rash, eschar, pneumonitis, meningitis, and disseminated intravascular coagulation, leading to severe multiorgan failure in untreated cases (Walker *et al.*, 1988).

Indirect immunofluorescent antibody assay (IFA) using polyclonal or monoclonal antibodies has identified four *O. tsutsugamushi* prototypes: Gilliam, Karp, Kato, and Boryong (Tamura *et al*., 1984; Murata *et al*., 1986; Yamashita *et al*., 1988; Chang *et al*., 1990). Also, variant serotypes have been reported in China, Japan, Thailand, and other Asian countries (Enatsu *et al*., 1999; Qiang *et al*., 2003; Blacksell *et al*., 2008). Some other strains such as the Yongworl, Pajoo, and Yonchon are identified as causative agents of *O. tsutsugamushi* in Korea (Chang *et al*., 1990; Seong *et al*., 1997; Shim *et al*., 2005; Yamashita *et al*., 1988).

56 kDa protein antigen is a primary antigen found in the sera of humans infected with scrub typhus. It displays type-specific antigenicity and is abundant in the outer membrane protein of *O. tsutsugamushi* (Ohashi *et al.*, 1988). This antigen plays an important role in the antigenic variants of *O. tsutsugamushi* and in dividing this pathogen into immunological serotypes (Hanson, 1985). Therefore, we investigated phylogenetic relationships within *O. tsutsugamushi* using this antigen protein sequence as a marker (Stover *et al*., 1990; Ohashi *et al*., 1992; Tamura *et al*., 2000).

Virulence or pathogenicity is related to the ability of microbe to induce disease (Hong *et al.*, 2005). The virulence factors have been identified on many other bacteria, including genes important for establishment of infection, survival, and replication within the mammalian host: *dnaA*, of the initiator protein of replication (Ishigo-Oka *et al.*, 2001); *virB8*, of the type IV secretion system (Baron, 2006); *tolR*, of the autotransporter family (Lazzaroni *et al.*, 2002); outer membrane protein *trxA* (Akif *et al*., 2008) ; superoxide dismutase *sodB* (Heinzen *et al*., 1992); translocation machinery component *secF* (Matsuyama *et al*., 1992); purine nucleotide synthesis protein *purC* (Ebbole and Zalkin, 1987); metabolic protein *tpiA* (McKnight *et al*., 1986); integral membrane protein *mviN* (Rudnick *et al*., 2001); energy metabolism enzyme *mdh* (Lin *et al*., 2004); *corC*, which is involved in magnesium and cobalt efflux (Wang *et al.*, 2004); *aas*, which is involved in escape membrane functioning (Hell *et al.*, 1998); heat shock protein *hscA* (Campos-Garcia *et al*., 2000); and ATP-dinucleoside polyphosphate hydrolase producer *secA* (Zimmer et al., 2008).

This is the first phylogenetic analysis of *O. tsutsugamushi* virulence genes using isolates from Korea. The result of phylogenetic analysis with virulence genes of *O. tsutsugamushi* provided important information of study on virulence of *O. tsutsugamushi*.

Materials and Methods

Bacteria

In this experiment, we used *O. tsutsugamushi* Boryong, Yonchon,

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Kuroki, Pajoo, Yongworl, Gunpo, and 3 human isolates (Deajeon03-01, Wonju03-01, and Muju03-01). We obtained *O. tsutsugamushi* Boryong and Yonchon from Seoul University Medical College, Korea, Kuroki from National Institutes of Infectious Disease, Japan. Pajoo, Yongworl, Gunpo, and 3 human isolates were isolated from patient blood that came from different locations (Daejeon, Wonju, and Muju, respectively) by our laboratory in 2003. Additionally, we used other *Rickettsia* to analyze the sequence from the NCBI.

Animals

Six-week-old male BALB/c inbred mice (Nara Biotech, Korea) were inoculated intraperitoneally (i.p.) with either 7×10^5 or 7×10^4 ICU of *O. tsutsugamushi* Deajeon03-01, Wonju03-01, or Muju03-01. After infection, the mice were monitored for 17 days, 4 mice per each strain. The mice were kept in biosafety level 3 animal facilities, where they received water and food ad libitum. Approval for animal experiments was obtained from the institutional animal welfare committee.

DNA extraction and PCR cloning

We obtained genomic DNA from *O. tsutsugamushi* species using the Genomic DNA Isolation kit (Promega, USA) according to the manufacturer's instructions. Every set of primers was designed based on sequences from the *O. tsutsugamushi* Boryong genome sequence (Cho *et al.*, 2007). With one set of primers for each gene, we analyzed 8 strains of *O. tsutsugamushi*. All PCR amplifications were performed using Ex *Taq* (TaKaRa, Japan). The primer sequences, annealing temperatures, and product lengths are listed in Table 1.

Determination of DNA gene sequences and phylogenetic analysis

The PCR products were purified using a quick spin purification kit (Amersham Pharmacia, USA) and were sequenced with an automated gene sequencer (ABI PRISM 377, Perkin-Elmer). The retrieved sequences were compared to those available in nucleic acid databases using the BLASTN database (National Center for Biotechnology Information) with BLAST to determine the closest relatives (Altschul *et al.*, 1997). The newly determined sequences were aligned with their closest relatives using the CLUSTAL X software program, and their phylogenetic relationship was analyzed using the software MEGA 4.0 with neighboring-joining method and the same parameters for the genetic distance calculation (Tamura *et al.*, 2007). Bootstrap values were calculated out of 1,000 replicates.

Statistical analysis

We compared the survival periods of the groups of infected mice using the log-Rank test. The two-tailed test was used to identify statistical significance to a 95% confidence interval. SAS software (version 9.1) was used for all statistical analysis.

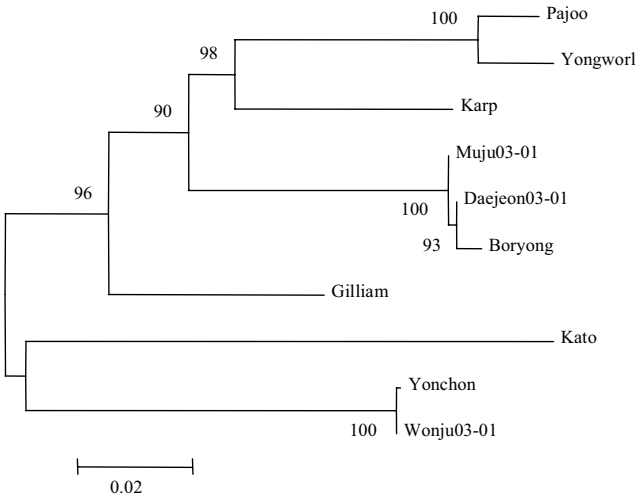

Fig. 1. Genetic diversity of *O. tsutsugamushi* strains, including isolates Deajeon03-01, Wonju03-01, and Muju03-01, based on the 56 kDa protein sequence. GenBank accession no. of each strain is GQ495610, GQ495611, and GQ495609, respectively. The number at the nodes indicates the bootstrap value. The bar shows the genetic distance of 0.02.

Results and Discussion

Differing fatality rates the *O. tsutsugamushi* strains Boryong strains; Deajeon03-01, and Muju03-01

Using the CLUSTAL X and MEGA 4.0 program, we clustered the *O. tsutsugamushi* reference strains Boryong, Karp, Gilliam, and Kato, Korean isolates Pajoo, Yongworl, and Yonchon, and our Deajeon03-01, Wonju03-01, and Muju03-01 isolates with the 56 kDa protein sequence from the NCBI. As a result, we identified Deajeon03-01 and Muju03-01 as Boryong strains and Wonju03-01 as a Yonchon strain (Fig. 1). Every 4 BALB/c mice were inoculated with the same concentration of each isolate to compare the fatality rate. After infection with 7×10^5 ICU of Deajeon03-01, 2 out of 4 mice died on the 8th day post-infection, and the remaining two mice died on the 9th day post-infection. In the case of Wonju03-01, all 4 mice died from the 11th to the 13th day post-infection. However, Muju03-01 had not caused any subject fatalities by the 17th post-infection day, when we halted observation (Fig. 2A). Similar results were seen when mice were inoculated with 7×10^4 ICU of the isolates Deajeon03-01, Wonju03-01, and Muju03-01 (Fig. 2B). These results indicated that there were virulence genes than

Table 1. PCR primer sequences and annealing temperatures used in this study

Target gene	Sequence (5′→3′)	Annealing temperature (°C)	Product (bp)
dnaA	F : ATGAATACTAAGTATCCAAA R : AGTAGGAGTACTAACAAA	45	1,296
virB8	F : AAAAATAAAAAGAAAGTTGAACA R : ACTATAATCATCATCTATTCT	45	666
tolR	F : ATGGACAACAATACAATAAAT R : CTTGTGTTCATTACAGTTA	45	456
trxA	F : ATGACTAAAAATATTACAGAG A R : ATAATTATTAATCCAATCTATAATAT	45	324

F, forward; R, reverse

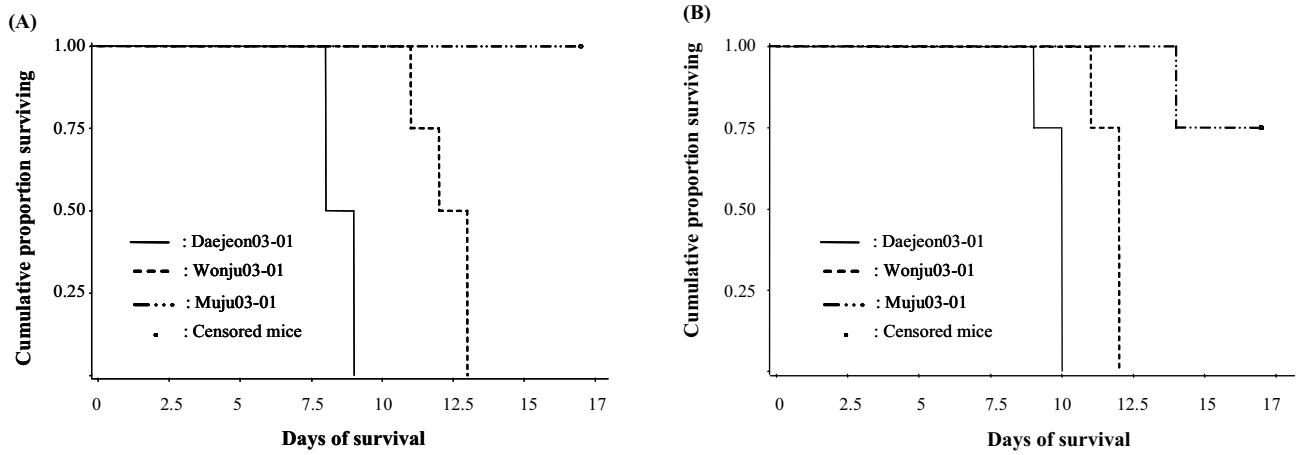

Fig. 2. Influence of high ICU (A) or low ICU (B) of *O. tsutsugamushi* Boryong isolates infection (P=0.0003 and P=0.0005, respectively) on the survival times of mice.

just the *O. tsutsugamushi* serotype influencing the fatality rates of infected mice.

Phylogenetic analysis of virulence genes in *O. tsutsugamushi*

The presence of virulence genes in *O. tsutsugamushi* was confirmed using PCR, based on other pathogens' virulence gene sequences from NCBI. After performing a sequence analysis of those genes, we did a phylogenetic analysis based on each of four virulence gene candidates. All four genes (*dnaA*, *virB8*, *tolR*, and *trxA*) were detected in *O. tsutsugamushi* Boryong and confirmed the existence of the same genes in other strains, including our isolates (data not shown). We

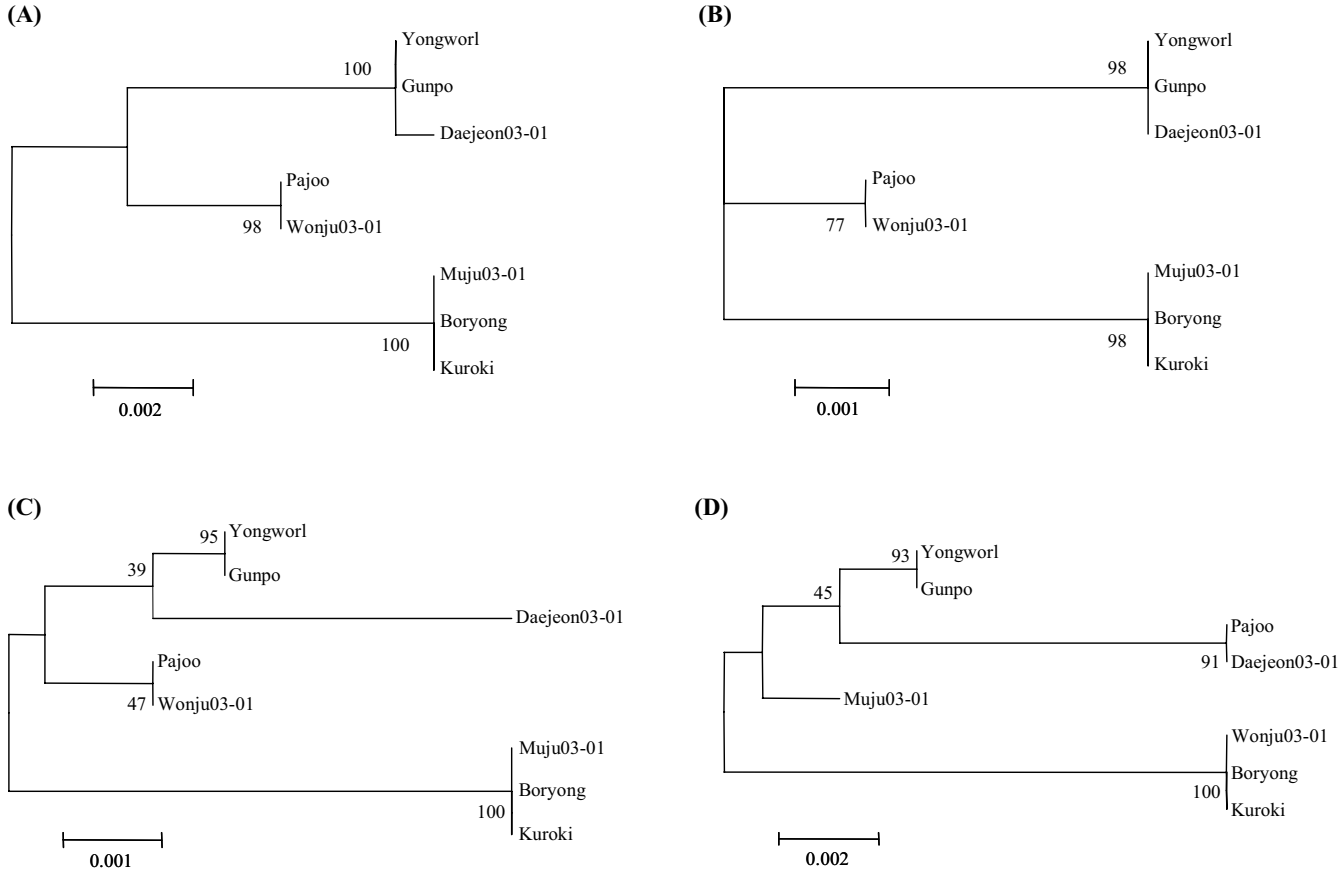

Fig. 3. Phylogenetic trees derived from various gene sequence data of *O. tsutsugamushi* strains. Bootstrap values at the nodes of the tree, which were based on replications, display the significance of these nodes. Horizontal bars represent sequence divergences. (A, *dnaA*); (B, *virB8*); (C, *tolR*), *and* (D, *trxA*). GenBank accession no. of 4 genes which we analyzed is from GU002367 to GU002398.

discovered these strains clustered differently with those virulence genes to 56 kDa protein sequence (Fig. 3A, B, C, and D). For this reason, we assumed, different sequences of the virulence genes of the isolates contributed to the differing fatality rates in the infected mice, regardless of the fact that they may have been in the same serogroup. On the other hand, we could not find any clustering trends among with other known virulence genes tested, such as *virB11*, *sodB*, *secF*, *purC*, *tpiA*, *mviN*, *mdh*, *corC*, *aas*, *hscA*, *secA*, *omp1*, *secA*, and *nuoF* (data not shown).

Comparison with virulence genes among Deajeon03-01 and Muju03-01

Figure 2 illustrates that the survival periods of subjects infected with the isolates Deajeon03-01, Wonju03-01, and Muju03-01 were significantly different, as were the fatality rates between high and low ICU concentrations of *O. tsutsugamushi* Boryong isolates Deajeon03-01 and Muju03-01 (P=0.0084 and P=0.01, respectively). A virulence gene and this function were identified through comparison of genome between virulence and avirulence strain the field of rikettsia study (Hong *et al*., 2004; Damon *et al*., 2008). The differences of *romp* and *rp084* gene in each other strains were showed relation of pathogenicity. Therefore, we analyzed the virulence gene sequences common to Deajeon03-01 and Muju03-01 to find the pathogenetic influence. Among those genes, we found different sequences in *trxA* (Fig. 4), *dnaA*, *virB8*, and *tolR* (data not shown). *Trx*A, known as the thioredoxin fold, maintains cellular redox status, along with other intracellular redox-regulating molecules. As the result of this *trxA* gene sequence analysis between Deajeon03-01 and Muju03-01, we found that 4 nucleotides differed (nucleotide positions 38 {A→G}, 55 {T→G}, 90 {T→C}, and 276 {G→A}), and the amino acid position 12, 19 changed (Asn → Ser and Phe → Leu, respectively) after the amino acids were modified. We also found divergence in nucleotide sequence among the genes; *dnaA* has 22, *virB8* has 6 and *tolR* has 4 divergent sequence. Based on sequence analysis of these virulence genes, we classified the *O. tsutsugamushi* genotype not only by 56-kDa protein sequence but also by virulence genes. Further studies are needed to reveal the relationship between each pathogenic gene or combination of genes and the pathogenicity of *O. tsutsugamushi*. Such a study could provide the detailed molecular mechanism of virulence gene functioning in *O. tsutsugamushi*.

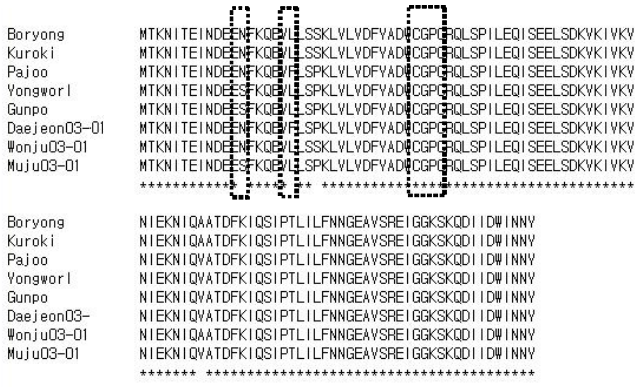

Fig. 4. Alignment of deduced amino acid sequences of the *trxA* gene of *O. tsutsugamushi* strains. Cys-Gly-Pro-Cys is highly conserved in the *trxA* gene family.

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