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 disease (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 Wiseman, 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 Yongwol, 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-Okada 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 (Akii 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); aac, 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,
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 7×10^4 or 7×10^5 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 DNA extraction and PCR cloning kit (Amersham Pharmacia, 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.

**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.

**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 7×10^4 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 7×10^5 ICU of the isolates Deajeon03-01, Wonju03-01, and Muju03-01 (Fig. 2B).

These results indicated that there were virulence genes than