Comparative Analysis of Nucleotide Sequences of Orientia tsutsugamushi in Different Epidemic Areas of Scrub Typhus in Shandong, China

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Abstract. Scrub typhus, caused by Orientia tsutsugamushi, is a zoonosis that spreads rapidly in Shandong, China. To investigate the molecular characterization of O. tsutsugamushi in new and old epidemic areas in Shandong Province, we compared the genetic relationships of O. tsutsugamushi between Linyi and Tai’an districts, typical old and new epidemic areas in Shandong, respectively. O. tsutsugamushi was detected in blood from 12 of 16 patients in Linyi and in eschar from 3 of 4 patients in Tai’an; 17 of 128 rodents were found to be infected with O. tsutsugamushi in Linyi, and 4 of 68 rodents were found to be O. tsutsugamushi-positive in Tai’an. The results indicated less genetic variation in O. tsutsugamushi between the new and old epidemic areas, and the Sdu-1 type, similar to Japan Kawasaki, was the main genotype of O. tsutsugamushi in Shandong Province.

INTRODUCTION

Scrub typhus, also known as tsutsugamushi disease, is a zoonosis that is endemic mainly in the Pacific region of Asia. Classified in the genus Rickettsia, its etiologic agent is now identified as Orientia tsutsugamushi, a Gram-negative obligate intracellular bacterium. Clinical manifestations of the disease range from mild fever with few other symptoms such as enlarged lymph nodes, skin rash, splenomegaly, and hepatomegaly to a fatal syndrome characterized by multiple-organ failure. Scrub typhus can be treated effectively with tetracycline or doxycycline.

Epidemic foci of scrub typhus have been an expanding from south to north China in the past 20 years. Before 1986, scrub typhus was found only south of the Yangtze River, with the main epidemic season in summer. After 1986, however, scrub typhus was detected in northern China, with autumn and winter as the main epidemic seasons. In Shandong Province, the first scrub typhus outbreak occurred in Linyi District in 1986. In 1988, the disease appeared in Jinan District. Before 1996, many cases were reported only from Linyi and Jinan districts, and no cases were found elsewhere in Shandong Province. However, there was a scrub typhus epidemic in Jining District in 1996, and the disease was found in Yantai District in 1997 and in Weifang District in 2000. In Autumn of 2000, 65 scrub typhus cases, including 2 deaths, were diagnosed in Tai’an District. Therefore, the “old” epidemic areas of scrub typhus in Shandong are the areas where scrub typhus cases were first diagnosed and reported from 1986 to 1996, whereas the “new” epidemic areas are those areas where scrub typhus cases were first identified after 1996.

Lying north of the Yangtze River, Shandong Province is located between latitudes 34°25′ and 38°23′ north and longitudes 114°35′ and 122°43′ east. It is a warm-temperate zone with a continental monsoon climate. Average annual temperature ranges from 11–14°C, and precipitation ranges from 550–950 mm per annum. Linyi, a major agricultural area in Shandong, is a typical old epidemic area of scrub typhus in Shandong after its first outbreak in 1986, whereas Tai’an District, in the northwest of Linyi District, had its first outbreak of the disease in the autumn of 2000.

To identify the genetic characterization of O. tsutsugamushi in both new and old epidemic areas in Shandong Province, to provide evidence for disease control and prevention for local communities, Linyi and Tai’an districts—the representatives of old and new epidemic areas, respectively—were selected as study areas. Molecular biology techniques and epidemiological methods were adopted to analyze and compare the nucleotide sequences of O. tsutsugamushi isolated from patients and rodents in the 2 epidemic areas of scrub typhus.

MATERIALS AND METHODS

Diagnostic criteria for scrub typhus. Diagnostic criteria for scrub typhus are as follows:

- field exposure history 1–3 weeks prior to symptoms;
- sudden high fever accompanied by characteristic eschar or ulcer;
- enlarged lymph nodes, skin rash, splenomegaly, or hepatomegaly;
- agglutination titer > 1:160 in the Weil–Felix test using the OX-K strain of Proteus mirabilis; and
- a 4-fold or more rise of antibody titer against O. tsutsugamushi in the indirect immunofluorescence antibody assay (IFA).

Scrub typhus can be diagnosed when a patient has 3 of the items described above. Such diagnostic criteria are the national criteria in China and are followed by all physicians.

Specimen sources. In Linyi and Tai’an districts, acute-phase blood specimens from the scrub typhus patients were collected before antibiotic treatment from September 2004 to December 2006. During the convalescence phase, the spontaneously desquamated eschars were also collected from patients. In Autumn and Winter, the epidemic seasons of scrub typhus in Shandong, rodents in the above 2 study areas were captured in patients homes and wild in fields. The captured
rodents were examined for infection species, and then spleen tissue was removed from each rodent, using procedures. All specimens were stored at −70°C.

Primers. According to the references, primers—including a pair of outer primers (P34, P55) and a pair of inner primers (P10, P11)—were chemically synthesized by Invitrogen Biotechnology (Shanghai, China). The *O. tsutsugamushi*-specific primers were designed based on the nucleotide sequence of a mature 56-kilodalton surface protein in *O. tsutsugamushi* Gilliam. The outer primers were P34, 5'-tca age tta tgt tta tgt gaa tgt tcg g-3', and P55, 5'-agg gat ccc tgc tgt tgt cta tta ggc-3'. Inner primers were P10, 5'-gat cca gtc ctc tca ggc atc ccg aca gat gca cta tta ggc-3'.

Nested PCR. DNA was extracted from the peripheral blood and eschar of scrub typhus patients and from the spleen tissue of rodents by the phenol/chloroform/isoamyl alcohol method. Extracted DNA was finally dissolved in sterilized ultrapure water and stored at −20°C and then used as template for the primary PCR.

The primary PCR amplification was performed by outer primers. The PCR mixtures were composed of 5.0 μL of 10× Ex Taq Buffer (Mg2+ plus), 4.0 μL of dNTP mixtures (each 2.5 mM), 2.5 μL of each primer (10 μM), 1.0 μL of DNA template, 0.25 μL of Ex Taq (5 U/μL; TaKaRa Biotechnology, Dalian, China), and 34.75 μL of ultrapure water added to a final volume of 50 μL. The primary PCR program consisted of 1 cycle of 5 minutes at 94°C, and 30 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 2 minutes, an extension step at 70°C for 2 minutes, and an additional extension at 72°C for 10 minutes. Nested PCR was performed using the same conditions as the primary PCR. The primary PCR products as template DNA were re-amplified with the primary primers. Among the 16 peripheral blood samples from patients from Linyi, 12 of them contained the specific bands, as expected, indicating that these blood specimens were *O. tsutsugamushi*-positive. Three of the 4 eschar specimens of patients from Tai’an were infected with *O. tsutsugamushi*. In Linyi, the overall infection rate was 13.28% among captured rodents, and positive rates of *O. tsutsugamushi* in *A. agrarius* and *C. tyiton* were 31.91% and 2.53%, respectively. No infection of *O. tsutsugamushi* was detected in *R. norvegicus*. In Tai’an, the overall *O. tsutsugamushi* infection rate was 5.88% in rodents; 12.5% in *A. agrarius*, 10.00% in *M. musculus*, but 0% in *R. norvegicus* (Table 1).

Sequencing. All nested PCR products of eschars (J-1, J-2, J-4), rodents (08P, 53P, 55P, 56P) from Tai’an, and patient blood samples (B-21, B-53, WG-2, FC-5, B-7, B-56, B-47, B-2, B-17, B-31, B-50, B-14) from Linyi were purified and sequenced. Several nested PCR products from rodents in Linyi were selected for purification and sequencing, including 87P and 10P from *A. agrarius* and 7P and 9P from *C. tyiton*. Nucleotide sequences of the 2 Shandong strains were submitted to

<table>
<thead>
<tr>
<th>Species of rodents</th>
<th>Linyi District</th>
<th>Tai’an District</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. captured</td>
<td>No. positive</td>
</tr>
<tr>
<td><em>Cricetulus tyiton</em></td>
<td>79</td>
<td>2</td>
</tr>
<tr>
<td><em>Apodemus agrarius</em></td>
<td>47</td>
<td>17</td>
</tr>
<tr>
<td><em>Rattus norvegicus</em></td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td><em>Mus musculus</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>128</td>
<td>17</td>
</tr>
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</table>

RESULTS

Specimens of patients and rodents. Overall, 16 peripheral blood specimens from Linyi District and 4 eschar specimens from Tai’an District were collected form scrub typhus patients. In the epidemic seasons of scrub typhus, a total of 128 rodents were captured in Linyi, including 79 *Cricetulus tyiton* (greater long-tail hamster), 47 *Apodemus agrarius* (striped field mouse), and 2 *Rattus norvegicus* (brown rat). Among 68 rodents captured in Tai’an, 34 were *R. norvegicus*, 24 were *A. agrarius*, and 10 were *Mus musculus* (house mouse) (Table 1).

Detection of *O. tsutsugamushi* by nested PCR. DNA extracted from all patients’ samples collected in Linyi and Tai’an Districts was amplified by nested PCR using the *O. tsutsugamushi*-specific primer pairs. Among the 16 peripheral blood samples from patients from Linyi, 12 of them contained the specific bands, as expected, indicating that these blood specimens were *O. tsutsugamushi*-positive. Three of the 4 eschar specimens of patients from Tai’an were infected with *O. tsutsugamushi*. In Linyi, the overall *O. tsutsugamushi* infection rate was 13.28% among captured rodents, and positive rates of *O. tsutsugamushi* in *A. agrarius* and *C. tyiton* were 31.91% and 2.53%, respectively. No infection of *O. tsutsugamushi* was detected in *R. norvegicus*. In Tai’an, the overall *O. tsutsugamushi* infection rate was 5.88% in rodents; 12.5% in *A. agrarius*, 10.00% in *M. musculus*, but 0% in *R. norvegicus* (Table 1).

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<table>
<thead>
<tr>
<th>Table 1</th>
<th>Rodents captured during the epidemic season for scrub typhus and Orientia tsutsugamushi infection status, Shandong, China</th>
</tr>
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<tbody>
<tr>
<td>Species of rodents</td>
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GenBank and were assigned accession numbers DQ489310 (WG-2) and EF543196 (55P).

**Sequence analysis.** All sequences of *O. tsutsugamushi* isolated from Shandong and other published in NCBI were aligned and compared using the program MEGA3.1. Some sequences were trimmed, whereas 458 bp—the informative region—was conserved and used for nucleotide sequence analysis.
Sequences of 12 *O. tsutsugamushi* isolated from patients were compared with sequences of 4 *O. tsutsugamushi* isolated from rodents in Linyi District. Homologous comparison showed that all of these isolates had 99.3–100% identities. In Tai’an District, the sequences of 3 *O. tsutsugamushi* isolated from patients were compared with the sequences of 4 *O. tsutsugamushi* isolated from rodents there, and the homologies among them were 74.3–100% identities. When the sequences of 16 *O. tsutsugamushi* samples isolated from Linyi District were compared with the sequences of 7 *O. tsutsugamushi* samples from Tai’an District, there were high identities among these isolates (from 73.9–100%). The 55P and 56P isolates from Tai’an had 92.5% identity with each other. However, other *O. tsutsugamushi* samples isolated from Linyi and Tai’an districts formed another category, with 99.3–100% identity among them. In addition, 55P and 56P from Tai’an shared 73.9–76.9% identity with other Shandong isolates.

The phylogenetic relationships among these *O. tsutsugamushi* isolates from Shandong and other *O. tsutsugamushi* obtained from GenBank were investigated. The Sdu-1 strain in NCBI was the nucleotide sequence of WG-2 isolated from this study, and the Sdu-2 strain was the sequence of 55P isolated from this study as well. As indicated in Figure 1, the phylogenetic tree based on the 56-kDa gene sequence homologies was divided into two branches. The majority of Shandong *O. tsutsugamushi* isolates, including the Sdu-1 strain, formed an independent lineage distinct from other *O. tsutsugamushi*, together with Kawasaki, Kanda, Taguchi, and Oishi strains from Japan. There were 95.5–95.9% identities between Sdu-1 and the 4 Japan strains. TW461 and Hualien-2 strains isolated from Taiwan, Yonchon from Korea, and Sxh951 from Shanxi Province, China, belonged to another clade. The 56P and 55P (Sdu-2) isolated from A. agrarius in Tai’an formed a separate clade. The first branch was constructed with the clades described above. The second branch was divided into 3 clades (Figure 1). One clade was formed by UT177 and Karp isolated in Thailand, Boryon from Korea, and Kuroki from Japan. Kato, isolated in Thailand, formed a second clade. TA763, TA686, and TA716, all isolated in Thailand, were located in the third clade.

**DISCUSSION**

This study suggested that the infection rate of *O. tsutsugamushi* among rodents was 13.28% in Linyi and 5.88% in Tai’an District. This indicated that *O. tsutsugamushi* infection among rodents was serious in Shandong Province, China. Previous sero-epidemiological investigation showed that *A. agrarius* was the major host of scrub typhus in Shandong, and *C. tyiton* might also be a reservoir in Shandong, China. Similar to that study, our molecular epidemiologic study indicated that both of *A. agrarius* and *C. tyiton* were reservoirs of scrub typhus in Shandong Province, with *A. agrarius* as main host. Moreover, we also identified *M. musculus* infected with *O. tsutsugamushi*, indicating that the house mouse might also be a reservoir of scrub typhus in Shandong Province.

Homologous comparison among all the *O. tsutsugamushi* isolates from Linyi and Tai’an districts showed that 55P and 56P had 73.9–76.9% identities with other Shandong isolates. The 55P and 56P isolated from Tai’an were in the same category, with 92.5% identity with each other. Other Shandong *O. tsutsugamushi* isolates belonged to another category and had 99.3–100% identities among them. The Sdu-1 strain enrolled in GenBank, the representative of major Shandong *O. tsutsugamushi* isolates, was distributed widely in Shandong Province (Figure 1). In addition, the Sdu-2 strain appeared in Tai’an, a new epidemic focus of scrub typhus in Shandong. At the molecular level, it was proved that the new epidemic area of scrub typhus in Shandong was closely related to the old epidemic area.

Phylogenetic analysis was conducted with all the *O. tsutsugamushi* isolates from Shandong, China, and other *O. tsutsugamushi* obtained from GenBank. As indicated in Figure 1, except for 55P and 56P, the other Shandong *O. tsutsugamushi* isolates were in the same clade, including Sdu-1 (GenBank accession no. DQ489310). 55P and 56P isolates from Tai’an formed a separate clade. However, all of these Shandong *O. tsutsugamushi* isolates were located in the same branch. It showed that there was only less genetic variation of *O. tsutsugamushi* in Shandong Province. The Sdu-1 type of *O. tsutsugamushi* was isolated not only from all patients’ blood and eschar but also from rodents, including *A. agrarius*, *C. tyiton*, and *M. musculus*. The tree showed distinctly that Sdu-1 and Oishi, Taguchi, Kanda, and Kawasaki types isolated from Japan were in the same lineage. Furthermore, there were high identities (95.5–95.9%) between Sdu-1 and the 4 types from Japan; Kawasaki type was the representative of the 4 *O. tsutsugamushi* types. Our study indicated that the Sdu-1 type, similar to Japan Kawasaki, was the main genotype of *O. tsutsugamushi* in Shandong, China.

In conclusion, our study showed that there was only less genetic variation of *O. tsutsugamushi* in both new and old epidemic areas in Shandong Province. The Sdu-1 type, similar to Kawasaki type in Japan, was the main genotype of *O. tsutsugamushi*. For prevention and control of scrub typhus in this area, more attention should be paid to surveillance of Sdu-1 type *O. tsutsugamushi*, both in human beings and rodents.

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