# CHARACTERIZATION OF *ORIENTIA TSUTSUGAMUSHI* STRAINS ISOLATED IN SHANDONG PROVINCE, CHINA BY IMMUNOFLUORESCENCE AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) ANALYSES

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**Abstract.** In order to identify the characteristics of the Sta56 gene of the 23 isolates of *Orientia (O.) tsutsugamushi* isolated in Shandong Province, indirect immunofluorescence assay (IFA) was used to identify the gene type of 23 strains *O. tsutsugamushi* isolated from scrub typhus patients, chigger mites, and rodents. Restriction fragment length polymorphism (RFLP) analysis was also used to analyze the restriction profiles of the Sta56 gene PCR amplification products of the 23 isolated strains of the *O. tsutsugamushi*; the results were compared with those acquired by nested PCR. By IFA, 21 of the 23 isolates belonged to the Gilliam type, and 2 to the Karp type. Using RFLP analysis, 21 strains had similar restriction profiles to the Japan Kawasaki strain, but they had no restriction site Hha I, and thus had some difference in gene sequence compared with the Japan Kawasaki strain. The other 2 strains had similar restriction profiles to Karp. These results were identical to that acquired by nested-PCR. In Shandong Province, the gene types of epidemic *O. tsutsugamushi* strains were similar to the Japan Kawasaki type, but had some differences in gene sequence. In addition, Karp also existed.

## INTRODUCTION

Scrub typhus occurs mainly in Southeast Asia and also in the Indian subcontinent and Australia. *Orientia (O.) tsutsugamushi*, which is transmitted by infected larval chigger mites, is the causative agent of disease. Several antigenic types have been recognized in *O. tsutsugamushi*. Shishido (1962) first described antigenic diversity among Gilliam, Karp, and Kato strains, and Tamura *et al* (1984) later found other types, such as Shimokoshi, Kawasaki (Yamamoto *et al*, 1986), and Kuroki (Yamamoto *et al*, 1989; Ohashi *et al*, 1990a) strains. In China, before 1986, scrub typhus only appeared in southern China (South of the Changjiang River), such as Guangdong, Hainan, Guangxi, Fujian, Zhejiang, Yunnan provinces and was epidemic mainly in summer. The main serotypes of *O. tsutsugamushi* were Gilliam, Karp, and Kato strains. In 1986, scrub typhus was firstly reported in Shandong Province (North of the Changjiang River) by Yang *et al* (1987), and was very different compared with the disease reported in southern China, as it was mainly epidemic in autumn. In the present study, 23 strains of *O. tsutsugamushi* were successfully isolated from patients, field rodents, and larval chigger mites in new scrub typhus foci of Shandong Province. In order to identify the characteristics of the epidemic *O. tsutsugamushi* isolated, besides a serological method (IFA), nested PCR, and RFLP analysis were used to precisely identify the genotypes.

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## MATERIALS AND METHODS

### Isolation of *O. tsutsugamushi*

In the epidemic season, livers, spleens and kidneys of 3 to 5 living rodents of the same species as a pool, or 71~200 chiggers of the same species (after having been washed with physiological saline containing penicillin and streptomycin for 3 to 5 changes) as a pool, were respectively homogenized in 2 ml physiological saline to make a 10% homogenate. 0.5 ml blood of patients, or the homogenate of rodents' internal organs or chigger mites were injected respectively into the peritoneal cavities of 3 laboratory mice (0.5 ml/mouse) to isolate

*O. tsutsugamushi*. After having been inoculated, the passage mice received injections of cyclophosphamide 0.25 mg/g body weight on days 0, 5, 10 for immuno-suppressant treatment. The inoculated mice were observed daily and the surviving mice were autopsied at 12~14 days. Smears from the peritoneal fluid scrape, liver, spleen and kidney of the passage mice were made and stained with Giemsa to reveal *O. tsutsugamushi*. All negative specimens were blindly passaged three to four times in mice. A total of 23 strains of *O. tsutsugamushi* were isolated from scrub typhus patients, chigger mites, and host rodents in Fei County, and Zouping County, Shandong Province. The passage mice sera, livers, spleens and kidneys of the isolated strains were stored at -30ºC until use.

**Detection of antibodies to *O. tsutsugamushi* and identification of serotypes**

All the sera from the passage mice were kept at -30ºC until use. The IFA method and serotyping used was that described by Guo *et al* (1994). Briefly, yolk capsules of hen eggs were separately infected with standard Gilliam, Karp, and Kato strains (supplied by National Vaccine and Serum Institute, Beijing), and the homogenates were spotted onto slides and allowed to react with appropriately diluted sera. Then sheep anti-mouse IgG fluorescent antibody was used to reveal the existence of *O. tsutsugamushi* antibody.

**Primers**

According to the references (Stover *et al*, 1990; Ohashi *et al*, 1990b; 1992; Guo *et al*, 1997), primers, including group and type primer, were synthesized by Shanghai Sanggon Biological Engineering Technology and Service Corporation, corresponding to the nucleotide sequence of scrub typhus antigen 56-kDa (Sta56) surface protein gene, whose diversity determined antigenic variations of *O. tsutsugamushi*. The two group primers were as follows:

Primer 1: +5′-TAC ATT AGC TGC AGG TAT GAC-3′

Primer 2: -5′-AAT TCT TCA ACC AAG CGA TCC-3′

The five type primers were as follows:

Primer G: -5′-TGA GCA AGA ATA TCA GTA TC-3′

Primer Kp: +5′-CAG ACC TCA GCA GCA AGC AC-3′

Primer Kt: -5′-ATA CCG CTG AGG CAT AGG AG-3′

Primer Kw: +5′-ATG CTG CTA TTG ATA CAG GC-3′

Primer Kr: -5′-TTG CGC TTG TGC CTG AGG TA-3′

Primer 1 matched with primer G as Gilliam type primer pairs, the PCR product was 255bp; Primer 2 with primer Kp as Karp type primer pairs, the PCR product was 85 bp; Primer 1 with Primer Kt as Kato type primer pairs, the PCR product was 154 bp; Primer 2 with Primer Kw as Kawasaki type primer pairs, the PCR product was 195 bp; Primer 1 with Primer Kr as Kuroki type primer pairs, the PCR product was 268 bp.

**Reagents**

Taq DNA polymerase, dNTPs were obtained from TaKaRa Biotechnology (Dalian) Corp. The restriction endonucleases SnaB I, Hha I, and Hinf I were purchased from Promega. The pUC19DNA/Msp I (Hpa II) Marker was obtained from MBI Fermentas. The PCR cycle was purchased from Hangzou Dahe Corp. International reference strains (Gilliam, Karp, and Kato) were kindly provided by Beijing Military Medical Institute.

**DNA extraction**

DNA extraction was done according to Chen (2001), and was briefly as follows: (1) 0.5g of spleen was homogenized and added 1xTE buffer, centrifuged at 3,000*g* for 5 minutes, and the supernatant discarded; (2) added 400 μl lysis buffer [10 mmol/l Tris (pH8.0), 0.1 mol/l EDTA, 0.5%SDS], 10 μl proteinase K (20 mg/ml), 2 μl lysozyme (4 mg/ml), and incubated at 50ºC for 6 hours; (3) DNA was extracted with phenol/chloroform three times, then precipitated in ethanol, washed with 75% ethanol and dissolved in sterilized distilled water, stored at -20ºC, and used as template for the first PCR.

**The primary PCR amplification**

The primary PCR amplification was carried out by group primer 1 and primer 2. The reaction was performed in 50 μl containing 10xPCR buffer 5 μl, 15 mM $MgCl_2$ 2 μl; 10 mM dNTP 3 μl, Taq DNA polymerase (5U/ μl) 0.25 μl; 10 μM primer 1, 1 μl; 10 μM primer 2, 1 μl; template DNA 1 μl; 36.75 μl sterilized distilled water. The amplification program consisted of one cycle of 5 minutes at 94ºC, and 35 cycles of heat denaturation at 94ºC for 30 seconds, annealing at 56ºC for 1 minute, and extension at 72ºC for 1 minute. This

was followed by a final extension reaction of 5 minutes at 72ºC. Then the amplification products were electrophorized in 2% agar containing ethidium bromide, and observed under ultraviolet transillumination; when 317-332 base-pair-specific bands were detected, the samples were designated positive.

**Purification of amplification products**

Under ultraviolet transillumination, cut off the agar containing the 317-332 base-pair-specific bands, and then put the agar into purification tube (Ultrafree-DA; American Millipore Corp), centrifuged at 5,000*g* for 15 minutes, and the purified DNA was at the bottom of the retrieve tube, then precipitated in ethanol, washed with 75% ethanol and dissolved in sterilized distilled water, stored at -20ºC for nested PCR, RFLP, and sequencing analysis.

**Restriction endonuclease digestion**

The restriction endonucleases SnaB I, Hha I, and Hinf I, were selected, referring to the nucleotide sequence of prototype *O. tsutsugamushi* strains Gilliam, Karp, Kato, Kawasaki, and Kuroki. The restriction sites of SnaB I, Hha I, and HinfI in Gilliam, Karp, Kato, Kawasaki, and Kuroki strain are shown in Table 1.

Five microliters purified PCR product was mixed with 2 μl RE 10 x buffer, 5U restriction endonucleases, 0.2 acetylated BSA (10 μg/μl) in a volume of 20 μl reaction system and incubated at 37ºC for 3 hours. Three microliters of the digested samples were loaded onto 12% PAGE gels, and run vertically for 3 hours at 200V in 1xTBE buffer using electrophoresis apparatus (Beijing Liuyi Apparatus Corp), and then subjected to silver staining. The genotypes of the isolates to be detected were determined by comparing their digestion patterns with those of the prototype *O. tsutsugamushi* strains.

**Nested PCR**

The amplification conditions were same as the first PCR, but using the purified amplification DNA products as templates, and adding 2 μl type primer pairs. Then the amplification products were electrophorized in 12% PAGE as above. When the specific band corresponding to the type primer pairs (for example, 255bp band corresponding to the Gilliam type primer pairs) appeared, this sample was regarded as this type (Gilliam type) of *O. tsutsugamushi.*

## RESULTS

**Serotypes of *O. tsutsugamushi***

The results of serotype identification of the isolated strains were as follow: except that 2 strains isolated from *A. agrarius* (FXS4 strain) and *L. linhuaikongense* (LHGM2 strain) belonged to the Karp type, which the other 21 strains were all Gilliam type (Table 2).

**Results of primary PCR**

Twenty-three isolated strains and international reference strains (Gilliam, Karp, and Kato) were detected, and all specific bands appeared as expected.

**RFLP analysis**

The RFLP profiles of PCR products digested by three restriction endonucleases are shown in Table1 and Fig 1 (showing only 5 representative strains: B16 strain from patients, FXS2 strain from

Table 1
The restriction sites of restriction endonucleases SnaB I, Hha I, and Hinf I.

| Restriction endonucleases | Fragment length of prototype *O. tsutsugamushi* strains (bp) | | | | | Fragment length of strains isolated in Shandong Province (bp) | |
|---|---|---|---|---|---|---|---|
| | Gilliam | Karp | Kato | Kawasaki | Kuroki | Strains 1-21 | FXS4, LHGM2 strain |
| SnaB I | 165 | 171 | | 165 | 171 | 165 | 171 |
| | 161 | 161 | - | 152 | 161 | 152 | 161 |
| Hha I | 250 | 271 | 293 | 175 | 182 | | 271 |
| | 76 | 61 | 27 | 142 | 72 | - | 61 |
| | | | | | 66 | | |
| Hinf I | 195 | 201 | 189 | | | | 201 |
| | 107 | 107 | 86 | - | - | - | 107 |
| | 24 | 24 | 45 | | | | 24 |

Table2
Genotyping of *O. tsutsugamushi* isolated in Shandong Province by IFA and nested PCR.

| Source of *O. tsutsugamushi* | Number detected | Serotyping by IFA | | | Genotyping by nested PCR | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Gilliam | Karp | Kato | Gilliam | Karp | Kato | Kawasaki | Kuroki |
| Patients | 8 | 8 | | | | | | 8 | |
| *A. agrarius* | 4 | 3 | 1 | | | 1 | | 3 | |
| *L. scutellare* | 4 | 4 | | | | | | 4 | |
| *L. palpale* | 3 | 3 | | | | | | 3 | |
| *L. linhuaikongense* | 2 | 1 | 1 | | | 1 | | 1 | |
| *W. pacifica* | 2 | 2 | | | | | | 2 | |

*A. agrarius*, XDM2 strain from *L. scutellare*, FXS4 strain from *A. agrarius*, and LHGM2 strain from *L. linhuaikongense*). Of the 23 strains, 21 (Nos. 1-21) had the same RFLP profiles (Fig 1, B16, FXS2, and XDM2 strain represented the RFLP profiles of Nos. 1-21). Compared with the RFLP profiles of the prototype *O. tsutsugamushi*, the 21 isolates' RFLP profiles were very similar to those of the Japan Kawasaki strain. Their DNA amplification products were digested into two bands (165 and 152 bp) by Snab I (Fig 1a) and had no restriction site of Hinf I (Fig 1c), but the isolates in Shandong Province had no restriction site of Hha I (Fig 1b). From comparison of RFLP profiles, the other 2 strains (FXS4 and LHGM2 strain) were the same as the Karp type.

**Nested PCR**

The amplified products of 23 strains and 3 international reference strains were re-amplified by type primer pairs; all appeared as corresponding specific bands. Out of 23 strains isolated, 21 strains belonged to Japan Kawasaki type, and 2 strains (FXS4 and LHGM2 strain) belonged to Karp type (Table 2).

## DISCUSSION

The antigenic heterogeneity of *O. tsutsugamushi* has been studied in some reports (Guo *et al*, 1994; Chen, 2001), most of them using serological methods. As serological methods were limited by the availability of monoclonal antibodies to various antigens, and by the existence of cross-reaction among antigens, it was difficult to accurately identify the type of *O. tsutsugamushi* by serological methods, let alone find new strain types. In the present study, 21 strains were classified as Gilliam type by IFA. This may possibly be due to the nucleotide sequence in the open reading frame (ORF) region of the Kawasaki strain having higher homology to that of the Gilliam strain than to those of the other strains (83% in nucleotide) (Ohashi *et al*, 1992). As the Japan Kawasaki strain was not available for IFA antigen in our study, the results acquired were understandable. In this study, we used RFLP analysis, and combined it with nested PCR precisely to investigate the molecular biological connections among them. The results acquired by these two methods were identical. In conclusion, the gene types of the epidemic *O. tsutsugamushi* strains in Shandong Province were similar to the Japan Kawasaki type, but had some differences in nucleotide sequence; Karp also existed in rodents and chigger mites.

Pham *et al* (2001) detected *O. tsutsugamushi* by PCR/RFLP in eight of nine species examined in both endemic and non-endemic areas of scrub typhus in Oita Prefecture, Japan.The results indicated that *O. tsutsugamushi* was widely distributed in various trombiculid species, and that possible horizontal transmission of *O. tsutsugamushi* might exist among trombiculid species. In Shandong Province, we found *O. tsutsugamushi* in the dominant species, *L. scutellare* in the endemic season, and we also detected *O. tsutsugamushi* in *L. palpale, L. linhuaikongense,* and *W. pacifica,* which appeared in non-endemic seasons. Thus the results partly supported the above viewpoint.

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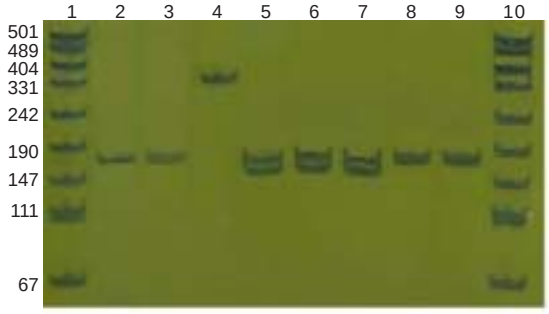


Fig 1a–RFLP profiles digested by SnaB I.

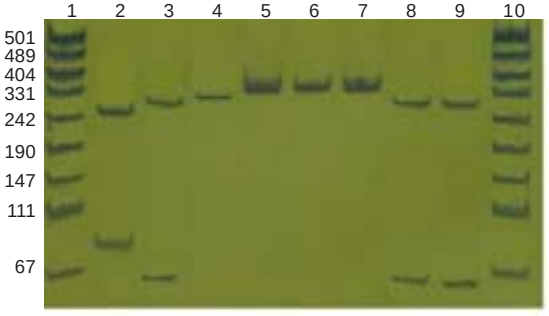


Fig 1b–RFLP profiles digested by Hha I.

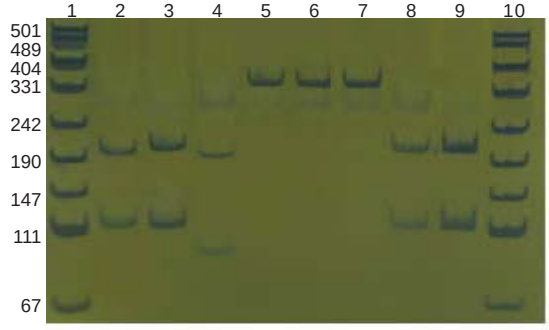


Fig 1c–RFLP profiles digested by Hinf I.

Fig 1–RFLP profiles of the sta56 gene PCR products of *O. tsutsugamushi* isolated in Shandong Province. 1,10 Marker pUC19DNA/Msp I (Hpa II); 2 Gilliam, 3 Karp, 4 Kato, 5 B-16, 6 FXS2, 7 XDM2, 8 FXS4, 9 LHGM2.

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