Laboratory Diagnosis and Genotype Identification of Scrub Typhus from Pinggu District, Beijing, 2008 and 2010

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Abstract. This study was conducted to determine the diagnosis and genotype of Orientia tsutsugamushi in Pinggu district, Beijing. Indirect immunofluorescence assay (IFA) was performed to detect O. tsutsugamushi-specific immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies. Nested polymerase chain reaction (PCR) and DNA sequencing analysis targeting the O. tsutsugamushi-specific groEL gene and 56 kDa protein gene were performed on whole-blood samples from scrub typhus patients. We confirmed that 47 patients were infected with scrub typhus in Pinggu district, Beijing. Representative sequences amplified by primers according to the groEL gene (BJ-PG-2008; GenBank accession No. JQ894502) and the 56 kDa protein gene (PG-56kDa; GenBank accession No. JX843795) both clustered with Kawasaki. PG-56kDa had sequence homology of 100% with TADY12-0308, shandong-XDM2, Neimeng-90, and sdu-1 and sequence homology of 96% with Kawasaki, Taguchi, Oishi, and Kanda. We confirmed the genotype of O. tsutsugamushi in Pinggu district, Beijing, as Kawasaki, and the patient in 2008 confirmed in this study was the first patient with confirmed scrub typhus in Beijing.

INTRODUCTION

Scrub typhus, also known as tsutsugamushi disease, is an acute, febrile illness that is caused by Orientia tsutsugamushi, which is an obligate intracellular bacterium that belongs to the family Rickettsiaceae in the order Rickettsiales. It is a zoonosis transmitted by infected larval trombiculid mites, and it is widespread in the Asia–Pacific region, including Afghanistan, China, Korea, the islands of the southwestern Pacific, and northern Australia. The clinical manifestations of this disease range from mild disease with symptoms of fever, rash, eschar, and lymphadenopathy to fatal disease. The disease can be treated effectively with doxycycline, azithromycin, or chloramphenicol.

In China, scrub typhus was only known to be endemic in areas south of the Yangtze River before 1986. Since the emergence of it in the northern provinces of Shandong and Jiangsu in 1986, epidemic range has enlarged to many other northern provinces in Tianjin, Shanxi, Heilongjiang, Jilin, Liaoning, etc. The epidemic foci of scrub typhus in China include three types: (1) foci located south of the Yangtze River (latitude about 30°N; mainly popular in the summer), including provinces of Hainan, Guangdong, Fujian, Zhejiang, Guangxi, Yunnan, Sichuan, Xizang, and Taiwan; (2) foci located north of latitude 40°N, including provinces of Heilongjiang, Jilin, and Liaoning (coastal areas and islands that border Russia and the Korean Peninsula), with a infection rate of 10% among the population; and (3) foci between latitudes 31°N and 40°N (autumn–winter-type scrub typhus, which occurs between September to December, with a peak in October and November) include the provinces of Jiangsu, Shandong, Tianjin, and Anhui. In northern China, the Leptotrombidium scutellare chigger is the predominant vector of O. tsutsugamushi, and host animals contain Apodemus agrarius, Rattus confucianus, R. norvegicus, Cricetulus trion, etc. In southern China, the L. deliens chigger is the predominant vector, and host animals mainly include R. losea, R. flavivermex, and A. agrarius.

Beijing is not an endemic area of scrub typhus in history. From 2008, patients with acute fever of unknown origin have been discovered in the district of Pinggu, and they also bear many other common characteristics of lasting fever for about 1 week before admission of hospital and varying degrees of rashes, which are ineffectively treated with cephalosporin but effectively treated with a kind of tetracycline named minocycline hydrochloride. Physicians diagnosed these cases as scrub typhus or typhus fever. Here, we report the laboratory diagnosis and genotype identification of scrub typhus in Pinggu district, Beijing, in 2008 and 2010.

MATERIALS AND METHODS

Geographical circumstance. Pinggu district is in northwestern Beijing, China (longitude: 116°55’ to 117°24’ E; latitude: 40°02’ to 40°22’ N). It is located in the intersection of Beijing, Tianjin, and Hebei provinces. Mountainous areas and semimountainous areas take up two-thirds of the district. The area has a warm, temperate, semihumid continental monsoon climate (average annual high temperature = 17.3°C, average annual precipitation = 633 mm).

Informed consent and permission to perform the study. This study was approved by the institutional review board and human research ethics committee of Beijing Center for Disease Prevention and Control (CDC). Informed oral consent was obtained from all study participants.

Sample collection. Patients admitted to Pinggu District Hospital and Pinggu District Traditional Chinese Medical Hospital in October of 2008 and from October to November of 2010 who were diagnosed with scrub typhus had 3–5 mL anticoagulant and 3–5 mL non-anticoagulant collected using vacuum blood collection tubes (BD Company, Franklin Lakes, NJ). According to the reports of epidemiological survey, 47 of these patients had fever plus one of the following symptoms: rashes, eschars or skin ulcer, or lymphadenopathy; therefore,

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the clinical diagnostic criteria for scrub typhus were met. However, there were five other patients with fever but without any other symptoms who were also diagnosed as scrub typhus.

**Nested polymerase chain reaction assay.** DNA was extracted from 0.2 mL whole blood of each sample using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The final volume of elution is 50 µL for each sample. Primers were synthesized by Shanghai Sangon Biotechnology Co. (Shanghai, China) and purified by polyacrylamide gel electrophoresis (PAGE).

A nested polymerase chain reaction (PCR) according to the groEL gene as recommended by the China CDC (Professor Lijuan, Zhang, unpublished data) and also reported by others8,9 was used as a detection method for *O. tsutsugamushi* for all samples. Primers Gro-1, 5′-AAGAAGGA/CGTACAAC-3′ and Gro-2, 5′-ACTTCA/CTAGCAC-3′ were used in the first PCR. Nested primers TF1, 5′-ATATATCACAGTACCTT GCAAC-3′ and TR2, 5′-GTTCCTACTTAGATGATCAT-3′ were used to amplify a 364-bp sequence from the scrub typhus group, and nested primers SF1, 5′-GATAAGAAGAAAAG CAATGATG-3′ and SR2, 5′-CACTTATTTAGGATT-3′ were used to amplify a 217-bp sequence from the spotted fever group (SFG) and typhus group (TG). The first round of PCR was performed in a 25-µL volume containing 5 µL template DNA, 1 µL each 10 pmol/µL primer (forward and reverse primers), 12.5 µL EX Taq HS (Takara, Kyoto, Japan), and sterile triple distilled water. The first and second rounds of PCR reactions were carried out under the following conditions: initial denaturation at 94°C for 5 minutes followed by 35 cycles, each consisting of denaturation at 94°C for 30 seconds, 55°C for 2 minutes, and final extension at 72°C for 7 minutes. The amplified PCR products were electrophoresed by the QIAxcel System; 17 PCR-positive samples of groEL gene were amplified by PCR according to the 56 kDa protein gene.

**Nucleotide sequences and phylogenetic analysis.** PCR products were sent to Shanghai Sangon for sequencing with a 3730 DNA analyzer (Applied Biosystems, Foster, CA). The retrieved sequences were made by basic local alignment search tool (BLAST) using the database of the National Center for Biotechnology Information (NCBI) to determine the closest relatives. Phylogenetic analysis among sequences detected in the present study and sequences registered in GenBank was performed using MEGA 5.05 software for the neighbor-joining method. Bootstrap analysis was performed 1,000 times to increase phylogenetic reliability. The phylogenetic analysis was performed with nucleotide sequences of the *O. tsutsugamushi* groEL gene and the 56 kDa gene as described by others.8,12

**Immunofluorescence assay.** We obtained acute-phase anticoagulated serum from each of 52 patients and six convalescent-phase sera. *O. tsutsugamushi* antigen slides were from Scimedx Company. Sera from patients and healthy controls were diluted from 1:32 in phosphate-buffered saline (PBS) with 3% non-fat powdered milk in twofold increments; 50 µL diluted serum were added to each well, and slides were incubated at 37°C humidified environment for 1 hour. Then, slides were washed with PBS containing 0.05% Tween-20. Next, fluorescein isothiocyanate (FITC)-conjugated anti-human–immunoglobulin M (IgM; Novus Biologicals, Littleton, CO) and FITC-conjugated anti-human–IgG (Sigma-Aldrich Co., St. Louis, MO) were added. Slides were incubated at

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**Figure 1.** PCR amplification results of groEL gene of samples from some scrub typhus patients in Pinggu.
37°C humidified environment for 1 hour and washed with the same wash buffer. An immunofluorescence assay (IFA) result was considered positive if any of the following were detected: (1) positive antibody titers ≥ 1:32 for IgM or ≥ 1:64 for IgG, (2) seroconversion, or (3) fourfold or more increase in titers in paired serum samples.

RESULTS

Epidemiological data. The first confirmed patient of scrub typhus in Pinggu in 2008 was a man of 59 years who settled in Pinggu more than 10 years ago from Anhui province. Clinical manifestation of the patient was fever and lymphadenopathy, with the highest temperature of 39°C. The epidemiological data of 46 patients confirmed by both IFA and PCR methods in 2010 were as follows: 47.8% (22/46) of patients were male, and 52.2% were female (24/46). Patients ages ranged from 17 to 77 years, with a median age of 57 years. The age distribution was as follows: 2.2% (1/46) of patients were below 19 years, 26.1% (12/46) were 20–49 years, 63.0% (29/46) were 50–69 years, and 8.7% (4/46) were above 70 years; 69.6% (32/46) of patients were farmers, 10.9% (5/46) were retired, and 19.5% (9/46) were at home or had other occupations. The duration time was from October 9th, 2010 to November 16th, 2010.

Clinical manifestation. Of the patients, 98.1% (51/52) had fever, and the average highest body temperature was 39.0°C. The duration of fever was 1–15 days (average = 5 days). Other major clinical manifestations included shivers (73.1%; 38/52), headache (67.3%; 35/52), rash (82.7%; 43/52), eschar or skin ulcer (73.1%; 38/52), and lymphadenopathy (7.7%; 4/52).

Nested PCR. PCR according to groEL gene using DNA extracted from 52 patients’ sera revealed that 47 patients’ samples contained O. tsutsugamushi DNA (Figure 1). The positive rates in 2008 and 2010 were, respectively, 100% (1/1) and 90.2% (46/51). Amplifications of SFG and TG Rickettsia sequences were negative of all samples. Seventeen samples amplified by primers according to the 56 kDa TSA gene of O. tsutsugamushi were all positive (Figure 2).

IFA. All blood samples of 52 clinically diagnosed patients and 20 healthy persons were tested. None of the healthy persons had serum antibodies to O. tsutsugamushi (IgM titer < 1:32, IgG titer < 1:64) (Figure 3). IgM and IgG antibody titers of the patient detected in 2008 were 1:64 and 1:128 for acute-phase serum and 1:128 and 1:512 for convalescent-phase serum. The positive rates of IgM and IgG in 2010 were 90.2% (46/51) and 88.2% (45/51). Forty-seven serum samples from patients in 2008 and 2010 were laboratory-confirmed cases that had positive antibody (Figure 4) and positive PCR results. Three blood samples from patients were laboratory
diagnosed as negative, with negative results of both PCR and antibody; two blood samples from patients were antibody-positive but PCR-negative, and they were laboratory-suspected cases. All six paired serum samples had a fourfold or more increase of IgG antibody, including samples from the patient in 2008.

**Sequencing and phylogenetic analysis.** All retrieved sequences of groEL gene amplification products were the same. Representative sequence BJ-PG-2008 was submitted to GenBank and got an accession number of JQ894502. The sequence was made BLAST on the NCBI database, and the similarity was 100% with Kawasaki genotype. Phylogenetic relationships of *O. tsutsugamushi* groEL gene sequences detected in this study and other groEL sequences of *Rickettsiae* available from GenBank were investigated. The phylogenetic tree shows that BJ-PG-2008 formed a cluster with the genus *O. tsutsugamushi* which was separate from the other cluster of the genus *Rickettsia* (Figure 5). All 17 sequences of 56 kDa gene amplification products were the same. Representative sequence PG-56kDa was submitted to GenBank and got an accession number of JX843795. The sequence was made BLAST on the NCBI database, and the similarity was 100% with TADY12-0308 (GenBank accession No. JX202589), shandong-XDM2 (GenBank accession No. DQ514320), Neimeng-90 (GenBank accession No. DQ514325), and sdu-1 (GenBank accession No. DQ489310) and 96% with Kawasaki (GenBank accession No. M63383), Taguchi (GenBank accession No. AF173038), Oishi (GenBank accession No. AF173037), and Kanda (GenBank accession No. AF173038). The phylogenetic tree based on the 56 kDa gene of the PG-56kDa sequence and

![Figure 5. Phylogenetic tree based on the nucleotide sequences of *O. tsutsugamushi* 56 kDa genes.](image-url)
other representative sequences of the genus *O. tsutsugamushi* was constructed by the neighbor-joining method with MEGA software by a bootstrap value of 1,000. The phylogenetic tree illustrates that PG-56kDa is closest with TADY12-0308, Shandong-XDM2, Neimeng-90, and Sdu-1. It forms a cluster with the Kawasaki strain, whereas there are other clusters of Gilliam, Karp, Kato, Kuroki, and Saitama (Figure 6).

**DISCUSSION**

The district of Pinggu is in the northwestern suburb of Beijing, and it is in the intersection of Beijing, Tianjin, and Heibei provinces. Both the provinces of Tianjin and Hebei had outbreaks of scrub typhus in the 1990s. In addition, considering the geographical circumstance of Pinggu district as warm temperature, rainfall, and mountain areas predominantly depending on agriculture, there was a great possibility of the existence of scrub typhus in Pinggu district before 2008. However, there were no cases of scrub typhus before 2008 in Beijing according to the database of China Information System for Diseases Control and Prevention. A patient as a confirmed case of scrub typhus in 2008 in Pinggu district was the first patient in Beijing, and we also confirmed 46 patients as having scrub typhus, whereas results of PCR and antibody detection in the present study were negative.

In the present study, all 47 patients concluded to be infected with scrub typhus in Pinggu district had positive PCR and IFA results. Two patients with positive IFA results (IgM titer = 1:32, IgG titer = 1:64) but negative PCR results cannot be confirmed definitely because of the impact of the rheumatoid factor, which may causes false-positive IFA results; convalescent-phase serum should be tested to show fourfold increase of IgG antibody for confirmation. In addition, three patients were misdiagnosed by physicians as having scrub typhus, whereas results of PCR and antibody detection in the present study were negative.

The methods of PCR and IFA that we used in this study are specific and sensitive. However, as secondary-level general hospitals, both Pinggu District Hospital and Pinggu District Traditional Chinese Medical Hospital do not have specific diagnostic methods for scrub typhus, and the generally available diagnostic method is the Weil-Felix test, which has poor sensitivity and specificity. Differentiation of scrub typhus from other rickettsial diseases is very important. The family of Rickettsiae includes the genus *Rickettsia*, which consists of SFG and TG, and the genus *O. tsutsugamushi*. *GroEL* gene sequences have higher degrees of divergence among the *rickettsiae*, and they have been proven to be able to facilitate the rapid diagnosis of rickettsial diseases and differentiate rickettsial diseases from other acute febrile diseases.
the genus *Rickettsia* and genus *O. tsutsugamushi*; sequence BJ-PG-2008 formed a cluster with Kawasaki, whereas two other sequences BJ-TZ-OT-O39 (GenBank accession No. GU499952) and BJ-MY-OT-S49 (GenBank accession No. GU499948) obtained from GenBank, isolated from Beijing, and submitted by Zhang and others (unpublished data) were in the same cluster with Karp and Kato, which indicates that there may be different genotypes of *O. tsutsugamushi* in other suburbs of Beijing.

It has been reported that the virulence of *O. tsutsugamushi* is related to its serotype or genotype. However, serotyping is time- and labor-consuming, and it is limited by the need to include all prototype strains when testing a new strain. Therefore, molecular typing methods, particularly PCR amplification of the 56 kDa protein gene, followed by sequencing have been used extensively for investigations of genotypes of scrub typhus strains. There is time- and labor-consuming, and it is limited by the need to include all prototype strains when testing a new strain. Therefore, molecular typing methods, particularly PCR amplification of the 56 kDa protein gene, followed by sequencing have been used extensively for investigations of genotypes of scrub typhus strains.20–22, 56 kDa type-specific antigen, which contains both group- and type-specific epitopes, has proven to be more useful for differentiating between *O. tsutsugamushi* strains.23–25 A previous study by Yu and others showed that sequences from three samples were identical, with a homology of 96% with Kawasaki type, which was the same as the samples analyzed in this study. In the present study, sequences of the 56 kDa gene from Pinggu district form a cluster with Kawasaki, and patients diagnosed as having scrub typhus were not considered severely ill; they can be cured by minocycline within 7–10 days, which was the same as reported by Ohashi and others,20 Guo and others,20 and Yang and others27 (Kawasaki was a very virulent strain, and the clinical manifestation of this serotype was not severe).

Epidemiological data reported by Yu and others and data in this study show that most of the patients were farmers 20–69 years old and that the disease occurred during fall harvest season from early October to late November (at the same time as the autumn–winter scrub typhus in Shandong, Jiangsu, and Anhui). In northern foci of China, the *L. scutellare* chigger is the predominant vector of *O. tsutsugamushi*, and animals found to be infected with *O. tsutsugamushi* are *A. agrarius* and *R. confucianus*. In the suburbs of Beijing, rodents of *A. agrarius* and *R. confucianus* are common, as surveyed by Dou and others, but the dominant chiggers carried by rodents need to be investigated further.

In conclusion, our study confirmed that 47 patients had scrub typhus in Pinggu district in 2008 and 2010, and the genotype of *O. tsutsugamushi* in Pinggu district, Beijing, has highly homology with Kawasaki. However, more epidemiological study and additional investigation about the vector and host animals need to be carried out to confirm whether the district of Pinggu is an epidemic area of scrub typhus. Also, it is urgent to strengthen the diagnosis of scrub typhus by teaching physicians about scrub typhus and establishing specific diagnostic methods in hospitals.

Received December 4, 2012. Accepted for publication April 27, 2013.

Published online May 28, 2013.

Financial support: This study was supported by National Key Program for Infectious Disease of China Grant 2012ZX10004215.

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