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

WeiHong Li, Xiangfeng Dou, Liqin Zhang, Yanning Lyu, Zhizhong Du, Lili Tian, Xiuchun Zhang, Yulan Sun, Zengzhi Guan, Lijuan Chen, Xinyu Li, and Quanyi Wang*

Institute for Infectious Disease and Endemic Disease Control, Beijing Center for Disease Prevention and Control (CDC), Beijing, People's Republic of China; Institute for Infectious Disease and Endemic Disease Control, Pinggu County Center for Disease Control and Prevention, Beijing, People's Republic of China

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.¹ The clinical manifestations of this disease range from mild disease with symptoms of fever, rash, eschar, and lymphadenopathy to fatal disease.² The disease can be treated effectively with doxycycline, azithromycin, or chloramphenicol.³

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.^{4–7} 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.^{4–7} 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 triton*, etc.⁶ In southern China, the *L. deliense* chigger is the predominant vector, and host animals mainly include *R. losea*, *R. flavipectus*, and *A. agrarius*.⁶

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 semi-mountainous 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,

*Address correspondence to Quanyi Wang, Institute for Infectious Disease and Endemic Disease Control, Beijing Center for Disease Prevention and Control (CDC), Capital Medical University School of Public Health and Family Medicine, No. 16 He Pingli Middle Street, Dongcheng District, Beijing 100013, China. E-mail: bjcdcxm@126.com

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 μ L 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 others^{8,9} was used as a detection method for *O. tsutsugamushi* for all samples. Primers Gro-1, 5'-AAGAAGGA/CGTGATAAC-3' and Gro-2, 5'-ACTTCA/CGTAGCACC-3' were used in the first PCR. Nested primers TF1, 5'-ATATATCACAGTACTTTGCAAC-3' and TR2, 5'-GTTCTTAAGTATGTATCAT-3' were used to amplify a 364-bp sequence from the scrub typhus group, and nested primers SF1, 5'-GATAGAAGAAAAGCAATGATG-3' and SR2, 5'-CAGCTaTTTGAGATT-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- μ L volume containing 5 μ L template DNA, 1 μ L each 10 pmol/ μ L primer (forward and reverse primers), 12.5 μ L EX Taq HS (Takara, Kyoto, Japan), and sterile triple distilled water. The first round of PCR reactions was carried out under the following conditions: initial denaturation at 94°C for 5 minutes followed by 39 cycles, each consisting of denaturation at 94°C for 40 seconds, 45°C for 40 seconds, 72°C for 40 seconds, and final extension at 72°C for 4 minutes. The second round of PCR was conducted using 1 μ L first-round PCR product as the template under the following conditions: initial denaturation at 94°C for 5 minutes followed by 39 cycles, each consisting of denaturation at 94°C for 35 seconds, 56°C for 35 seconds, 72°C for 35 seconds, and final extension at 72°C for 4 minutes. The amplified PCR products were electrophoresed by QIAxcel System (Qiagen, Hilden, Germany), and a 15-bp/3-kb alignment marker (Qiagen, Hilden, Germany) was used.

A nested PCR according to the 56 kDa type-specific antigen (TSA) gene was used as a method for differentiation within *Orientia* strains. The assay was performed by a modification of the method described by Furuya and others.^{10,11} Nucleotide primers were based on the nucleotide sequence of the gene encoding the 56 kDa antigen of the Gilliam strain

of *O. tsutsugamushi*. Primers P34, 5'-TCA AGC TTA TTG CTA GTG CAA TGT CTGC-3' and P55, 5'-AGG GAT CCC TGC TGC TGT GCT TGC TGCG-3' were used in the first PCR. Nested PCR primers P10, 5'-GAT CAA GCT TCC TCA GCC TAC TAT AAT GCC-3' and P11, 5'-CTA GGG ATC CCG ACA GAT GCA CTA TTA GGC-3' were used in the second PCR amplification of the resulting 483-bp fragment. The first round of PCR was performed in a 25- μ L volume containing 5 μ L DNA, 1 μ L each 10 pmol/ μ L primer (forward and reverse primers), 12.5 μ L EX Taq HS (Takara, Kyoto, Japan), and sterile triple distilled water. Both 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, 72°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 μ L 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

FIGURE 1. PCR amplification results of *groEL* gene of samples from some scrub typhus patients in Pinggu.

FIGURE 2. PCR amplification results of 56 kDa 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 $\geq 1:32$ for IgM or $\geq 1:64$ 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

FIGURE 3. Negative IFA results of serum samples from healthy human control in Pinggu.

FIGURE 4. Positive IFA results of serum samples from patients with scrub typhus in Pinggu.

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. AF173039). 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.

FIGURE 6. Phylogenetic tree based on the nucleotide sequences of *Rickettsia groEL* genes.

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 Hebei provinces. Both the provinces of Tianjin and Hebei had outbreaks of scrub typhus in the 1990s.^{13,14} 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 in this district in 2010. A previous study by Fu and others¹⁵ confirmed an outbreak of scrub typhus in Pinggu district, Beijing, in 2009. As reported by physicians from Pinggu District Hospital in the work by Liu and others,¹⁶ 24 patients with scrub typhus in Pinggu district in 2008 were misdiagnosed with other diseases; this finding reflects the initial lack of recognition of scrub typhus by physicians and the lack of specific laboratory diagnostic tests for scrub typhus by hospitals. Misdiagnosis may also explain the

lack of reporting of scrub typhus before 2008 in the district of Pinggu, especially when the clinical manifestation is mild and unspecific.

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,¹⁷ which may causes false-positive IFA results; convalescent-phase serum should be tested to show fourfold increase of IgG antibody for confirmation.¹⁸ 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.¹⁷ The misdiagnosis caused by lack of specific diagnostic method and knowledge about scrub typhus is a common problem existing in China.^{7,16}

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.^{8,9} In the present study, the phylogenetic tree of *groEL* gene sequences forms two clusters of

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. GQ499952) and BJ-MY-OT-S49 (GenBank accession No. GQ499948) 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.¹⁹ 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^{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 others¹³ 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,²⁶ and Yang and others²⁷ (Kawasaki was a low virulent strain, and the clinical manifestation of this serotype was not severe).

Epidemiological data reported by Yu and others¹³ 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,²⁸ Jiangsu,²⁹ and Anhui⁷). 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*.⁵ In the suburbs of Beijing, rodents of *A. agrarius* and *R. confucianus* are common, as surveyed by Dou and others,³⁰ 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.

Authors' addresses: Weihong Li, Xiangfeng Dou, Yanning Lyu, Lili Tian, Xiuchun Zhang, Yulan Sun, Zengzhi Guan, Lijuan Chen, Xinyu Li, and Quanyi Wang, Institute for Infectious Disease and Endemic Disease Control, Beijing Center for Disease Prevention and Control, Beijing, People's Republic of China, E-mails: liweihong@yahoo.cn,

beijingcdc@163.com, lvyanning@yahoo.com, tianlili6324883@126.com, xchzhang@sina.com, sylan9999@yahoo.com.cn, guan_zzhi@163.com, bjcdcchlj@hotmail.com, bjcdcxy@163.com, and bjcdcxm@126.com. Liqin Zhang and Zhizhong Du, Institute for Infectious Disease and Endemic Disease Control, Pinggu County Center for Disease Control and Prevention, Beijing, People's Republic of China, E-mails: zllq197205@163.com and dzz1968@sohu.com.

REFERENCES

- Kelly D, Fuerst P, Ching W, Richards A, 2009. Scrub typhus: the geographic distribution of phenotypic and genotypic variants of *Orientia tsutsugamushi*. *Clin Infect Dis* 48: 203–230.
- Lee JH, Lee JH, Chung KM, Kim ES, Kwak YG, Moon C, Lee CS, 2013. Dynamics of clinical symptoms in patients with scrub typhus. *Jpn J Infect Dis* 66: 155–157.
- Fang YR, Huang ZH, Tu CY, Zhang LJ, Ye DQ, Zhu BP, 2012. Meta-analysis of drug treatment for scrub typhus in Asia. *Intern Med* 51: 2313–2320.
- Yu ES, Wu GH, 1995. Epidemiological types of *Tsutsugamushi* disease in China. *Chin J Zoon* 11: 16–19.
- Wu GH, 2000. Epidemiological study on scrub typhus in China—present status and prospects. *Chin J Infect Dis* 18: 142–144.
- Zhang LJ, Fu XP, Fan MY, 2005. Research on *Rickettsia* and epidemiological status of rickettsiosis in China. *Chin J Trop Med Parasitol* 3: 37–42.
- Zhang SY, Song HB, Liu Y, Li Q, Wang Y, Wu JB, Wan JF, Li GL, Yu CJ, Li XY, Yin WW, Xu Z, Liu B, Zhang Q, Wan KL, Li GC, Fu XP, Zhang JS, He JR, Hai R, Yu DZ, Walker DH, Xu JG, Yu XJ, 2010. Scrub Typhus in previously unrecognized areas of endemicity in China. *J Clin Microbiol* 48: 1241–1244.
- Lee JH, Park HS, Jang WJ, Koh SE, Kim JM, Shim SK, Park MY, Kim YW, Kim BJ, Kook YH, Park KH, Lee SH, 2003. Differentiation of *Rickettsiae* by groEL gene analysis. *J Clin Microbiol* 41: 2952–2960.
- Park HS, Lee JH, Jeong EJ, Kim JE, Hong SJ, Park TK, Kim TY, Jang WJ, Park KH, Kim BJ, Kook YH, Lee SH, 2005. Rapid and simple identification of *Orientia tsutsugamushi* from other group *Rickettsiae* by duplex PCR assay using groEL gene. *Microbiol Immunol* 49: 545–549.
- Furuya Y, Yoshida Y, Katayama T, Yamamoto S, Kawamura A Jr, 1993. Serotype-specific amplification of *Rickettsia tsutsugamushi* DNA by nested polymerase chain reaction. *J Clin Microbiol* 31: 1637–1640.
- Kim DM, Yun NR, Yang TY, Lee JH, Yang JT, Shim SK, Choi EN, Park MY, Lee SH, 2006. Usefulness of nested PCR for the diagnosis of Scrub Typhus in clinical practice: a prospective study. *Am J Trop Med Hyg* 75: 542–545.
- Enatsu T, Urakami H, Tamura A, 1999. Phylogenetic analysis of *Orientia tsutsugamushi* strains based on the sequence homologies of 56-kDa type-specific antigen genes. *FEMS Microbiol Lett* 180: 163–169.
- Yu CS, Zhang ZL, Luo YQ, Zhang Y, Liu ZY, Cong BQ, Liu ZY, Sun ZZ, Wang CL, Fan LH, Zhang CS, Chen LH, 1992. Report on first finding an epidemic of scrub typhus in north rural areas, Tianjin. *Chin J Epidemiol* 13: 212–215.
- Chen SL, Li GY, Li CM, Kong LY, Guo YX, Zuo PJ, Xu JH, Ji XY, 2000. First report of *Tsutsugamushi* disease in Hebei province: a preliminary study. *Chin J Vector Biol Control* 11: 213–215.
- Fu XP, Liu YY, Zhang BH, Wang JQ, Zhang JS, He JR, Zhang SY, 2011. Laboratory confirmation of scrub typhus outbreak in Pinggu district, Beijing. *Chin J Vector Biol Control* 22: 137–140.
- Liu YY, Zhang X, Bai XQ, Zhang XS, 2009. Analysis of 24 misdiagnosed cases of scrub typhus in Beijing. *Capital Medicine* 16: 54–55.
- La Scola B, Raoult D, 1997. Laboratory diagnosis of rickettsioses: current approaches to diagnosis of old and new rickettsial diseases. *J Clin Microbiol* 35: 2715–2727.
- Centers for Disease Control and Prevention, 2012. *CDC Health Information for International Travel 2012: The Yellow Book*. Available at: <http://wwwnc.cdc.gov/travel/yellowbook/2012/>

- chapter-3-infectious-diseases-related-to-travel/rickettsial-spotted-and-typhus-fevers-and-related-infections-anaplasmosis-and-ehrlichiosis.htm. Accessed December 3, 2012.
19. Ree HI, Kim TE, Lee IY, Jeon SH, Hwang UW, Chang WH, 2001. Determination and geographical distribution of *Orientia tsutsugamushi* serotypes in KOREA by nested polymerase chain reaction. *Am J Trop Med Hyg* 65: 528–534.
 20. Ohashi N, Nashimoto H, Ikeda H, Tamura A, 1992. Diversity of immunodominant 56-kDa type-specific antigen (TSA) of *Rickettsia tsutsugamushi*. *J Biol Chem* 267: 12728–12735.
 21. Lee YM, Kim DM, Lee SH, Jang MS, Neupane GP, 2011. Phylogenetic analysis of the 56 kDa protein genes of *Orientia tsutsugamushi* in southwest area of Korea. *Am J Trop Med Hyg* 84: 250–254.
 22. Fournier PE, Siritantikorn S, Rolain JM, Suputtamongkol Y, Hoontrakul S, Charoenwat S, Losuwanaluk K, Parola P, Raoult D, 2008. Detection of new genotypes of *Orientia tsutsugamushi* infecting humans in Thailand. *Clin Microbiol Infect* 14: 168–173.
 23. Ohashi N, Nashimoto H, Ikeda H, Tamura A, 1990. Cloning and sequencing of the gene (tsg56) encoding a type-specific antigen from *Rickettsia tsutsugamushi*. *Gene* 91: 119–122.
 24. Stover CK, Marana DP, Carter JM, Roe BA, Mardis E, Oaks EV, 1990. The 56-kilodalton major protein antigen of *Rickettsia tsutsugamushi*: molecular cloning and sequence analysis of the sta56 gene and precise identification of a strain-specific epitope. *Infect Immun* 58: 2076–2084.
 25. Manosroi J, Chutipongvivate S, Auwanit W, Manosroi A, 2006. Determination and geographic distribution of *Orientia tsutsugamushi* serotypes in Thailand by nested polymerase chain reaction. *Diagn Microbiol Infect Dis* 55: 185–190.
 26. Guo HB, Wu GH, Tang JQ, Li XF, Yu MM, Zhang Y, Pan XZ, Li YX, 1995. Finding out Kawasaki serotype *Rickettsia tsutsugamushi* by nested polymerase chain reaction in China. *Chin J Zoon* 11: 22–24.
 27. Yang LP, Zhao ZT, Liu YX, Feng YQ, Wang XJ, Li Z, 2006. Genotype identification and sequence analysis of *Orientia tsutsugamushi* isolated from Shandong area. *Chin J Epidemiol* 27: 1061–1064.
 28. Yang ZQ, Liu YX, Yu XM, Wu QY, Xing RY, 2000. Investigation on natural foci of autumn-winter type *tsutsugamushi* disease in Shandong province. *Chin J Epidemiol* 21: 283–286.
 29. Guo HB, Wu GH, Xu MH, Liu Y, Yu MM, Shen JZ, Bao MR, 1994. Studies on natural foci of *Tsutsugamushi* disease of the Autumn–Winter type in Jiangsu. *Chin J Epidemiol* 15: 27–30.
 30. Dou XF, Li YH, Li X, Lv YN, Li XY, Guan ZZ, Zhang XC, Wang XM, He ZY, Sun YL, Lin H, Tian LL, Liu Y, Wang QY, 2012. Monitoring results of plague in small mammals in Beijing. *Chin J Ctrl Endem Dis* 27: 101–103.