FOLLOW-UP ANALYSIS ON THE EPIDEMIC STRAINS OF *ORIENTIA TSUTSUGAMUSHI* IN THE FIRST OUTBREAK OF SCRUB TYPHUS IN HENAN PROVINCE, CHINA

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Abstract. To obtain knowledge of the genetic characteristics and types of the epidemic strains of *Orientia tsutsugamushi* in the first outbreak of scrub typhus in Henan Province, genus and type-specific primers were employed to amplify a fragment of the gene of 56 kDa protein. Serotyping demonstrated that, of the 19 patients [15 patients in recovery phase (10-40 days) and 4 of patients in acute phase (1-7 days)], 4 were infected with Gilliam type, 8 with Kato type, 6 with Karp type, and 1 with an unknown type. Successful genotyping was obtained for only 3 patients, indicating that 2 were infected with Karp type and 1 with Taiwan Kato type. Thus the outbreak of scrub typhus in Henan Province was caused by at least two epidemic strains.

INTRODUCTION

Scrub typhus is a disease of natural focus caused by *Orientia tsutsugamushi*. Mice are mainly the source of infection and chiggers are the vehicle of infection. This disease is mainly distributed in Asia-Pacific region (Aung-Thu *et al*, 2004). Presently, it is regarded as a significant risk factor for public health in Southeastern Asian Region and poses a threat to tourists (Walker, 2003). Before 1986, scrub typhus was mainly distributed in southern provinces and municipalities of China, including Hainan, Guangdong, Zhejiang, Guangxi, Yunnan, Sichuan, Hunan, Tibet, and Taiwan. Since 1990, it has rapidly expanded its region of infection, spreading to the north of the Yangtze River. Its prevalence has been successively found in Jiangsu, Shanxi, Shandong, Tianjin, and the northeastern region. Presently, it has been reported in 26 provinces and municipalities except Beijing, Qinghai, Inner Mongolia, Gansu, and Hubei in China (Fig 1). This disease is mainly characterized by non-specific clinical manifestation, such as high fever, headache, as well as overall soreness. It is often diagnosed as other infectious diseases, especially in the rural areas, leading to complications to multi-organs and increased mortality of patients (Thap *et al*, 2002; Li *et al*, 2005).Therefore, experts and scholars in China have strongly urged that scrub typhus be listed as an infectious disease requiring management (Chen, 2001). In autumn and winter of 2005, the first outbreak of scrub typhus occurred in Henan Province in the central plain of China. This paper reports the follow-up analysis of the epidemic strains of this outbreak.

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MATERIALS AND METHODS

Geographic features

Huaibin County, Xinyang city, is located

Fig 1–Prevalence areas of scrub typhus in China.

in the southeast of Henan Province (east of 115º25′E and north of 32º27′N) and at the northern foot of "Dabie" mountain with Anhui Province on its east. Its altitude is 34 m and has a continental climate with an annual average temperature of 17.4ºC and an annual average rainfall of 926 mm.The county consists of hillocks, plains and billabongs, and three rivers, Huai River, Hong River and Bailu River flow through. The epidemic areas were in the alluvions of Huai River and Hong River and the epidemic was spread by canals. The environment of villages was very dirty (Fig 2) with wild grass growing throughout the farmland and interfluves.

Outbreak overview and research subjects

Thirty-two patients with scrub typhus in the outbreak in Dadong Village, Huaibin County, Xinyang City, Henan Province from September 26-November 10, 2005 were recruited. The average age was 36.3 (3-75 years), including 11 males and 21 females. All patients consented to be investigated, have their blood collected and have their data published. The patients mainly showed fever, overall soreness, weakness, flatulence, poor appetite, and nausea in the prodrome, complicated with headache, and maculopapular rash in face and head, body, and then limbs (Fig 2). Some patients had vomiting, as well as swelling lymph nodes in postauricular and occipital lymph nodes. As scrub typhus was not suspected in the early phase of the outbreak, eschar was only found in 2 patients through inquiry of disease history (Fig 2). Patients were not sensitive to treatment with penicillin or ampicillin, and improved with cefoperazone, reduction of fever and detoxification, and through symptomatic management. Most patients were observed for 10-14 days. Some patients were discharged from the hospital before laboratory tests could be conducted for scrub typhus, and only 5 patients

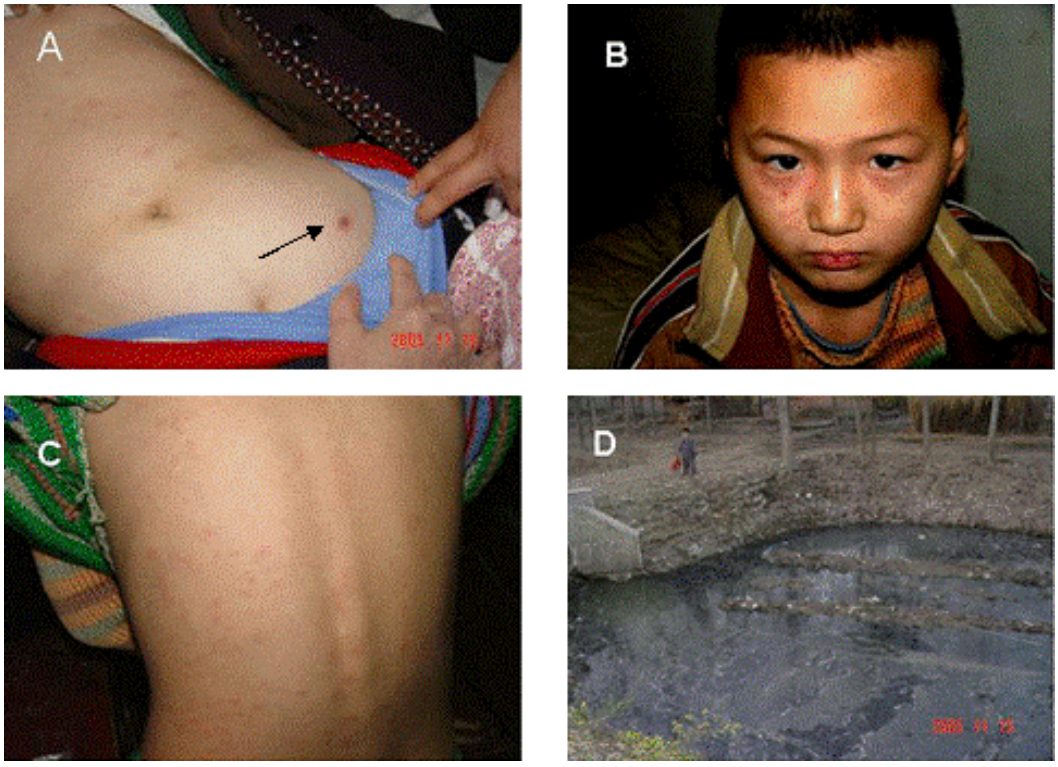

Fig 2 Rash (A, B, C) and eschar (indicated in A by arrow) of patients with scrub typhus, as well as peripheral environment of the outbreak (D).

provided samples for Weii-Felix test for *O. tsutsugamushi*. In the second blood collection, 19 whole blood samples were obtained, including 15 patients in recovery phase (10-40 days) and 4 patients in acute phase (1-7 days).

Serological test

Serological Weil-Felix reaction was conducted according to the conventional method (Zhang, 1998), using diagnosing reagents purchased from Shanghai Institute for Biological Products, OX2, OX19, and OXK (State Drug Approval No. S10820304, S10820305, and S10820312). Results of ≥1:160 were considered positive. Indirect immunofluorescent test (IFA) was conducted according to the method of Philip *et al* (1976), and antigen-glass slides of Gilliam, Karp, and Kato strains of *O. tsutsugamushi* were provided by WHO Cooperation Center of Rickettsiae (Mediterranean University, Marseille, France). Fluorescence-labeled anti-human antibodies (IgM and IgG) were purchased from Sigma. The results were considered positive when a titer of ≥1:80 was obtained. In IFA, the type was defined when homologous antibody titer was two times or more of that of heterologous antibody.

DNA extraction, PCR amplification and DNA sequencing

DNA was extracted from coagulated blood by using a kit purchased from Qiagen (Qiagen, Hilden, Germany). Genus-specific and type-specific primers of *O. tsutsugamushi* 56 kDa envelope protein gene were those of Liu *et al* (2004): genus-specific primer 1: 5′-TAC ATT AGC TGC AGG TAT GAC-3′; genus-specific primer 2: 5′-AAT TCT TCA ACC AAG CGA TCC-3′. Gilliam typing was conducted by combination of genus-specific primer 1 with

primer G (5′-TGAGCAAGAATATCAGTATC-3′) to amplify a 255bp fragment; Karp typing by combination of genus-specific primer 2 with primer Kp (5′-CAGACCTCAGCAGCAAGCAC-3′) to amplify a 85bp fragment; and Kato typing by combination of genome-specific primer 1 with primer Kt (5′-ATACCGCTGAGG CATAGGAG-3′) to amplify a 154 bp segment. The 25 μl reaction system included 10 μl of DNA template, 1 μl (20 pM) to each primer, 0.5 μl of dNTP (10 mM) mixture (dATP, dCTP, dGTP, dTTP), 0.5 μl (2 U) of Taq DNA polymerase (SBS Biotechnology), and 2.5 μl of buffer. DNA of Karp strain of *O. tsutsugamushi* (provided by Fujian CDC, China) was used as positive control, and sterile distilled water as negative control. PCR reaction was conducted in Bioer-TC-48/H(t) Thermal Cycler (Hangzhou Bioer Technology), with the following conditions : denaturation for 15 minutes at 94ºC, 39 cycles of 40 seconds at 94ºC, 50 seconds at 56ºC, and 1 minute at 72ºC, with a final incubation for 7 minutes at 72ºC.

PCR products were purified by PCR-purifying kit (WATSON Biotechnologies), inserted into pMD19-T cloning vector (Takara), and transfected into *E. coli* (Tianwei Biotechnology). White colonies were selected and the plasmids were sent to Shanghai Shenggong Biotechnology for sequencing. Sequence analysis was conducted using NCBI BLAST and DNAstar software.

RESULTS

Of the 19 samples, 15 patients were in the recovery phase with IFA titers ≥1:160 and a positive rate of 100%. The highest titer was 1:2560. Eight samples from healthy persons gave negative results. Of the 4 samples from patients in the acute phase (patients in the late stage of the outbreak), one had a titer of ≤1:80, and the other three were of ≥1:160. Samples were collected again from those 4 patients in the recovery phase (weeks 3-4), and examined by the Weil-Felix test, and the results showed increases of 4 times or more. Of the 15 patients in recovery phase, OXK serum antibody titers were all ≥1:160 using the Weil-Felix test, with a positive rate 100%, and the highest titer of 1:2560. Five patients showed OXK serum antibody titer 4 times higher than those in acute phases. Serotyping showed that 8 patients were of Kato type, 6 of Karp type, 4 of Gillian type and one was of an unknown type.

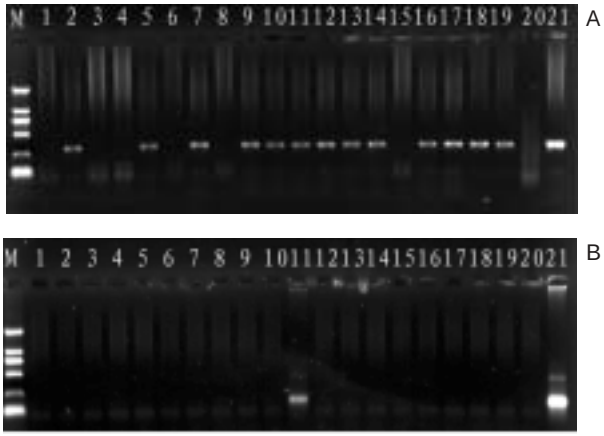

Fig 3–PCR amplification by using genus-specific primers for 56 kDa protein gene (A) and for Kato type (B). Protocols for PCR amplification are described in Materials and Methods. M: size Markers of 2,000 bp, 1,000 bp, 750 bp, 500 bp, 250 bp and 100 bp. 1-19: samples. 20: negative control. 21: positive control.

PCR detection of *O. tsutsugamushi* produced fragments of 317-332 bp in 13 samples, including 3 samples from patients in acute phase (Fig 3A). PCR for Kato type showed a DNA fragment of 154 bp for sample from No. 11 patient (Fig 3B). PCR amplification of other types gave negative results.

DNA sequencing of PCR products of samples from patients No.16 and 17 showed 100% homology with sequence of CDC Karp-type 56 kDa specific antigen gene of *O. tsutsugamushi*. Nucleic acid and their deduced amino acids showed 100% homology be-

tween No. 16 and 17 patients. Direct sequencing of PCR product of sample from patient No. 11 showed 100% homology with sequence of CDC Taiwan strain Kato type 56 kDa specific gene of *O. tsutsugamushi.*

DISCUSSION

It is recognized that there are 8 serum types of scrub typhus. Epidemic strains of scrub typhus in China include Karp, Gilliam, and Kato types. However, new epidemic strains have appeared in recent years in China, such as Kawasaki epidemic in Jiangsu and Shandong Province (Guo *et al*, 1995; Liu *et al*, 2004) which was first found in Japan. A closely-related Yonchon strain from Korea was also found in Shanxi Province (Chen, 2000). From serotyping, this study showed that the first outbreak of scrub typhus in Henan Province was due to epidemic strains of Karp, Kato, and Gilliam types. As serotyping often shows some deviation due to cross reactions between different types of antigens, as well as the limited range of antigens used, PCR amplification and DNA sequencing were also used and demonstrated that the outbreak was due to the presence of at least two epidemic strains of Karp and Kato type. This was the first outbreak reported in Henan Province in China and further research is needed to understand the source of infection and whether this region has other epidemic types.

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