ORIGINAL ARTICLE

Molecular characterization of *Orientia tsutsugamushi* causing scrub typhus among febrile patients in north-central Bangladesh

M. M. Al Amin[1], S. K. Paul[1], M. S. Aung[2], A. Paul[1], M. A. Aziz[1], N. A. Khan[1], A. K. M. F. Haque[1], F. Ahamed[1], A. Melan[1], S. R. Sarker[1], M. A. Hossain[3], S. Ahmed[1], S. A. Nasreen[4], N. Haque[1] and N. Kobayashi[2]

1) *Mymensingh Medical College, Mymensingh, Bangladesh,* 2) *Sapporo Medical University School of Medicine, Sapporo, Japan,* 3) *National Institute of Preventive and Social Medicine (NIPSOM), Dhaka, Bangladesh and* 4) *Sheikh Hasina Medical College, Jamalpur, Bangladesh*

Abstract

Scrub typhus is a mite-borne rickettsial disease caused by *Orientia tsutsugamushi,* which is endemic in Asia Pacific region. In this study, infection rate and molecular epidemiologic traits of *O. tsutsugamushi* was investigated in Mymensingh, located in north-central Bangladesh. Among the blood samples from 453 febrile patients who visited Mymensingh medical college hospital in 2018, the 47 kDa protein gene of *O. tsutsugamushi* was detected in 78 samples (17.2%) by nested PCR. Phylogenetic analysis of the *O. tsutsugamushi* 56 kDa protein gene (18 samples) revealed a predominance of Karp-related genotype (89%), while the remaining belonged to Gilliam genotype. Samples of the Karp-related genotype mostly clustered with those of China, Taiwan, Thailand and India, etc., in emergent subgroups clades 2 and 4, which were distinct from clade 1, including prototype Karp strains. Among the 18 samples, three variable domains (VD) of 56 kDa type-specific antigen had different types of sequence diversity; VDI contained two or three repeats of eight amino acid units, while VDII and VDIII had amino acid substitution, deletion or insertion. The present study documented a potentially high prevalence of genetically diverse *O. tsutsugamushi* in north-central Bangladesh.

Keywords: 56 kDa type-specific antigen, Bangladesh, *Orientia tsutsugamushi*, scrub typhus, Karp-related genotype
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Corresponding author: N. Kobayashi, Department of Hygiene, Sapporo Medical University School of Medicine, S-1 W-17, Chuo-ku, Sapporo 060-8556, Japan.
E-mail: nkobayas@sapmed.ac.jp

Introduction

Scrub typhus is an acute febrile illness caused by *Orientia tsutsugamushi,* an obligate intracellular bacterium which is transmitted by larval stages of *Leptotrombidium* mites [1]. This disease is estimated to threaten one billion people globally and causes illness in one million people each year, with variable mortality rate (0–70%) [2,3]. Distribution of scrub typhus has been restricted to the so-called Tsutsugamushi triangle, which includes most of the South and Southeast Asian countries, generally showing a seroprevalence of up to 40% in the general population [4]. Outside Asia, an endemic focus of scrub typhus has been described in South America, in southern Chile [5].

Several genotypes (Gilliam, Karp, Kato, Shimokoshi, Kawasaki, TA763, Kuroki, etc.) are recognized among the strains of *O. tsutsugamushi* on the basis of the 56 kDa type-specific antigen (TSA), an immunodominant outer membrane protein unique to this bacterium. Karp is the most common genotype, responsible for about 50% of all scrub typhus cases [2,6]. These genotypes are defined by four variable domains (VD), I through IV, in the 56 kDa protein, which are responsible for the large degree of antigenic variation [7]. Clarifying genotypes as well as the genetic diversity of *O. tsutsugamushi* strains in endemic regions is essential for the development of rapid diagnostics and vaccines.

Bangladesh is located within the Tsutsugamushi triangle, and seroprevalence of *O. tsutsugamushi* was described as 23.7% throughout the country [8]. However, there has been no

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information regarding the genotype or genetic traits of *O. tsutsugamushi,* except for a single recent report in Chittagong (southeastern region) [9]. Accordingly, we conducted the present study in Mymensingh, in north-central Bangladesh, to elucidate prevalent genotypes of *O. tsutsugamushi* and their molecular epidemiologic features.

Materials and methods

In this study, we enrolled 453 febrile patients who visited Mymensingh Medical College Hospital, Mymensingh, north-central Bangladesh, from March 2018 to December 2018 (Fig. 1). These patients were suspected to have rickettsial illness because they had fever (axillary temperature >37.5°C) persisting for 5 days or more. Febrile cases with an established underlying aetiology other than rickettsial illness were excluded.

The approval of the institutional ethical committee was obtained before the beginning of the study. After obtaining informed written consent, 2 mL of venous blood was collected into an EDTA tube and stored at −20°C until further use.

By using DNA materials extracted from the blood samples, *O. tsutsugamushi* was detected by nested PCR targeting the 47 kDa antigen gene, as described previously [10]. The nucleotide sequence of the PCR product was directly determined by the Sanger method using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) on an automated DNA sequencer (ABI PRISM 3130). Sequence data were analysed for their identical or most similar sequence by BLAST search to identify *Orientia*. Further, for all the 47 kDa gene-positive samples, partial 56 kDa protein gene (approximately 750 bp), including VDI, II and III, was amplified by nested PCR using first-round primers (OtsuF: AATTGCTAGTGCAATGTCTG and 55R: AGCTGCTGCTGTGCTTGCTGCG) and second-round primers (primer E: GTTGGAGGAATGATTACTGG and 11R: CGACAGATGCACTATTAGGC), which were modified from those reported previously [10], except for primer E [11]. The nucleotide sequence of the partial 56 kDa protein gene was also determined directly from PCR products. A phylogenetic dendrogram of the partial 56 kDa protein genes determined in the present study and those retrieved from the GenBank database was constructed by the maximum likelihood method by MEGA6 software. Multiple alignment of the partial 56 kDa protein amino acid sequences was performed by the Clustal Omega programme (https://www.ebi.ac.uk/Tools/msa/clustalo/). Sequence identity among 56 kDa genes was calculated by the LALIGN programme (https://www.ebi.ac.uk/Tools/psa/lalign/). Eighteen partial 56 kDa protein gene sequences were deposited to GenBank under accession numbers MK617189 to MK617206.

Results

The *O. tsutsugamushi* 47 kDa gene was detected in 78 (17.2%) of 453 samples tested by initial nested PCR. Among the confirmed cases of scrub typhus, the most common manifestation was myalgia (61.5%), followed by headache (56.4%) and cough (56.4%), while eschar was present in only 14 cases (17.9%). Skin rashes, oliguria and jaundice were observed at low frequencies (10.3%, 10.3% and 9.0% respectively).

The sequences of 33 PCR products (118 bp) randomly selected from the positive samples were identical to the partial sequence of the 47 kDa protein gene of strain Karp (GenBank accession no. LS398548) (data not shown). Among the 33 samples demonstrating the 47 kDa protein gene, the partial 56 kDa TSA gene was amplified and sequenced for 18 samples. A phylogenetic tree of the 56 kDa gene was constructed for the 18 samples in the present study in Bangladesh, together with 65 reference strains (Fig. 2). Sixteen samples clustered within the Karp-related genotype, while two samples were within the Gilliam genotype. Strains of the Karp-related genotype were phylogenetically differentiated into four clades (1, 2, 3 and 4), and clade 4 was further divided into two subgroups (4a and 4b). The 16 Bangladeshi samples were assigned into clade 1 (one sample), clade 2 (six samples) and clade 4 (nine samples—three 4a and six 4b), then clustered with strains from China, Taiwan, Thailand, Cambodia and India. Within the partial 56 kDa TSA gene, sequence identity among the Karp-related genotype (different clades) was 88–94%, while the identity between the Karp-related genotype and the Gilliam genotype was 72–82% (Table 1).

Amino acid sequence diversity in the three VDs (I, II, III) of 56 kDa TSA was analysed by multiple sequence alignment of deduced amino acid sequences from 18 samples in Bangladesh and reference strains representing each genotype/clade (Supplementary Fig. S1). In the VDI of the Karp-related genotype, tandemly repeated eight amino acid (octapeptide)-sequence units (KV(T)KADSGG, EIKADSGG) were identified. Its repeat number was different depending on its clade: there were two repeats in clade 4b, three repeats in clade 4a, and two or three repeats in clades 1 and 2. The Gillian strain and OtMMC117 had similar two-repeat units, whereas OtMMC191 had only a part (six amino acids) of the unit. Within the VDII, three to five amino acid substitutions from the Karp strain were observed in clades 2, 4a and 4b, and were associated with deletion in clade 4a. VDIII of clade 2 was the most divergent from clade 1, having seven to nine amino acid difference

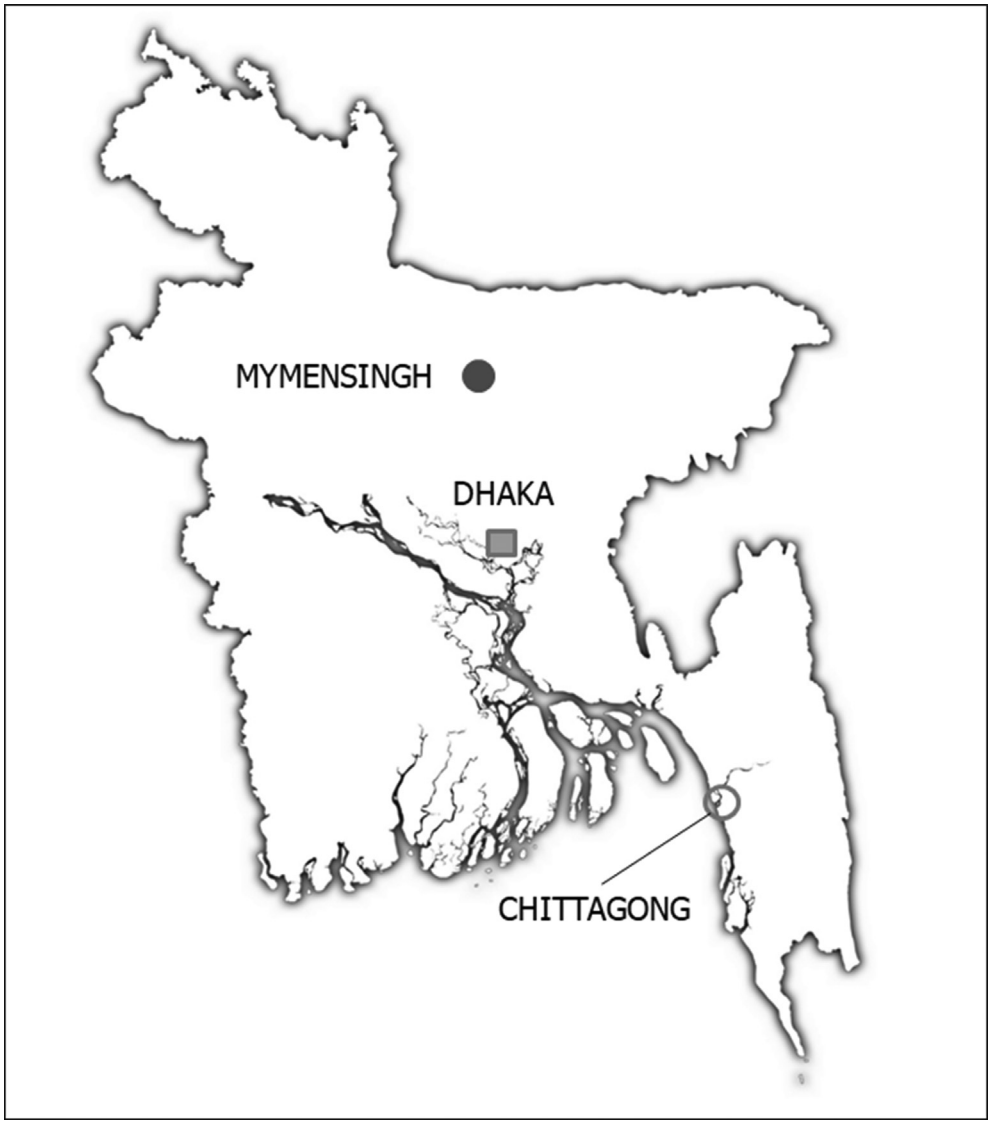

FIG. 1. Map of Bangladesh showing location of Mymensingh (solid circle, site of present study) and Chittagong (open circle) where *Orientia tsutsugamushi* was identified. Capital city of Dhaka is indicated by shaded square.

including two amino acid insertions, while the VDIII of clades 4a and 4b had a smaller number of amino acid substitutions or deletions compared to clade 2. Among reference strain Gilliam and two Gilliam genotype samples in this study, more sequence diversity was observed in VDI than in VDII and VDIII.

Discussion

In Bangladesh, the overall seropositive rate for *O. tsutsugamushi* has been described as 23.7%, with rates varying from approximately 15% to 45% among six study sites (excluding the north-central region), with the eastern and south regions (Comilla, Chittagong) showing the highest prevalence (more than 30%) [8]. The presence of *O. tsutsugamushi* genes in blood samples from febrile patients had been investigated in only one study in southeast Bangladesh (Chittagong), for a 10.9% detection rate [9]. In the present study in north-central Bangladesh, the genetically defined detection rate of *O. tsutsugamushi* was 17.2% among suspected cases of rickettsial illness. A prevalence of *O. tsutsugamushi* among febrile patients comparable to our study has been reported in eastern Taiwan (16%) [12], northern Thailand (21%) [13] and northeast India (Assam) (16%, IgM-positive cases) [14]. Accordingly, north-central Bangladesh is also considered to be an endemic area of scrub typhus in South Asia.

While distribution of *O. tsutsugamushi* genotypes is considerably different depending on geographical regions, the Karp-

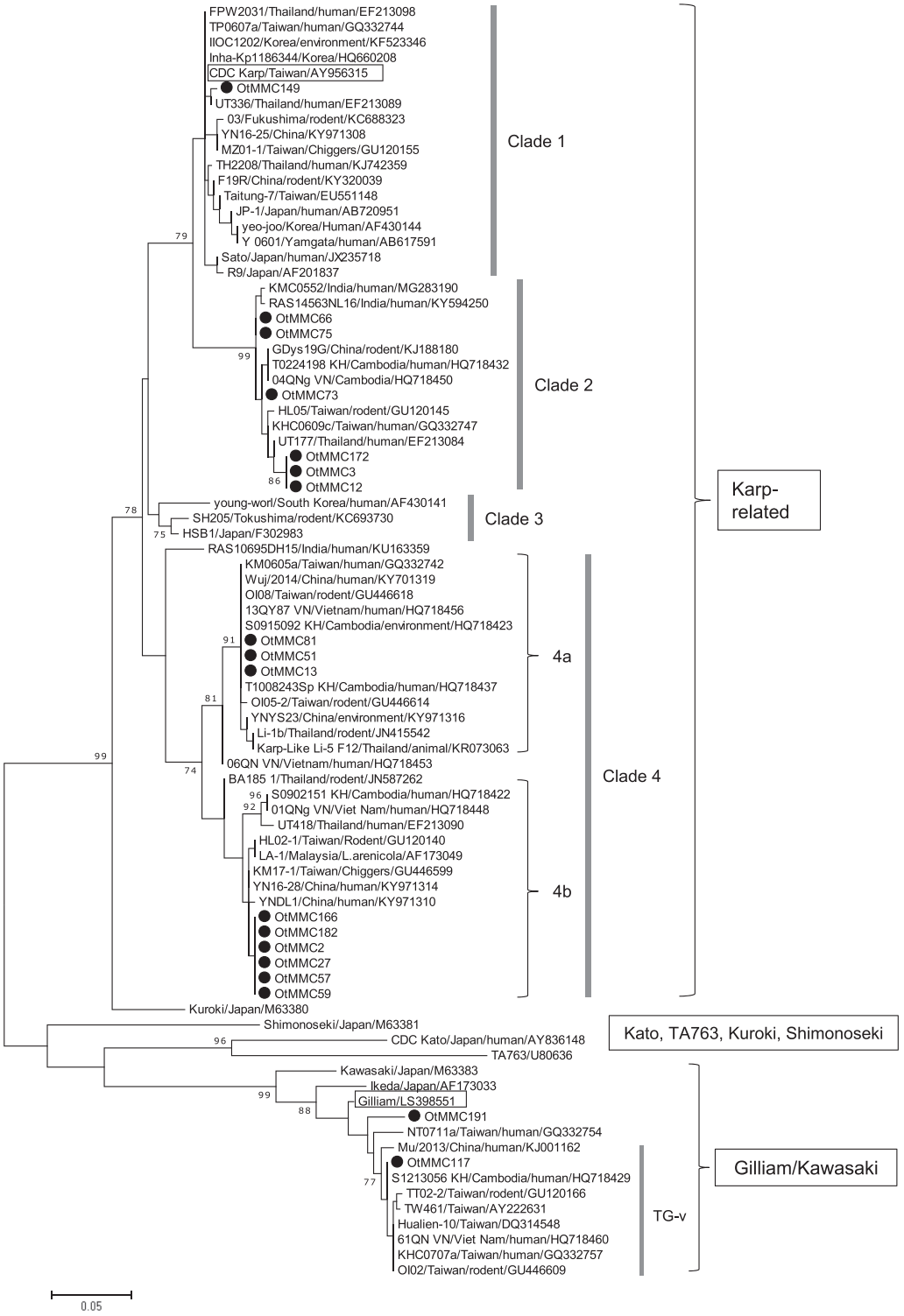

FIG. 2. Phylogenetic dendrogram of partial 56 kDa TSA genes generated for 18 samples in Bangladesh (closed circle) and 65 strains from diverse geographical locations, constructed by maximum likelihood method using MEGA6 software. Tree was statistically supported by bootstrapping with 1000 replicates, and genetic distances were calculated by Kimura 2-parameter model. Variation scale is described at bottom. Percentage bootstrap support is indicated by values at each node (values <75 are omitted). Prototype strains Karp and Gilliam are boxed. Major genotypes (Karp related, Gilliam/Kawasaki, Kato/TA763/Kuroki/Shimonoseki) and four clades, including subclades 4a and 4b of Karp-related genotype, are shown at right. TG-v, Taiwan Gilliam variant.

TABLE 1. Sequence identity (upper right, nucleotide; lower left, amino acid) of partial 56 kDa type-specific antigen containing variable domains (VDI, VDII and VDIII) among samples with Karp-related and Gilliam genotypes analysed in this study

		Sequence identity with strain/sample (%)						
Genotype/clade	Strain/sample	Karp	OtMMC149	OtMMC3	OtMMC51	OtMMC166	Gilliam	OtMMC191
Karp related, clade 1	Karp		93.4[a]	93.8[a]	88.4[a]	93.6[a]	82.0	75.0
Karp related, clade 1	OtMMC149	90.6[a]		92.8[a]	93.5[a]	92.6[a]	79.8	72.1
Karp related, clade 2	OtMMC3	89.4[a]	89.3[a]		88.7[a]	94.3[a]	82.3	75.5
Karp related, clade 4a	OtMMC51	83.2[a]	88.9[a]	83.2[a]		90.8[a]	79.9	73.1
Karp related, clade 4b	OtMMC166	89.8[a]	90.2[a]	89.4[a]	86.9[a]		82.0	75.0
Gilliam	Gilliam	73.3	70.9	72.9	70.4	72.5		88.0
Gilliam	OtMMC191	63.1	61.1	63.1	61.2	63.1	83.3	

[a]Identities present among Karp-related genotype samples.

related genotype has been described as being the most prevalent in East Asia (China, Japan), Southeast Asia (Philippines, Thailand, Vietnam, Cambodia) and northeast India (Assam) [2,14,15]. In Bangladesh, the predominance of the Karp-related genotype was observed in Chittagong [9], as well as in the present study in the north-central region, thus suggesting a widespread presence of the Karp-related genotype in this country. However, the Kato-like genotype was reported to be dominant in northeast India (Shillong), located near the border with Bangladesh, as well as in south India (Vellore) [16]. Thus, we suggest that Bangladesh may be at the westernmost limit of a Karp-dominant area.

In the present study, genetic diversity among the Karp-related strains in Bangladesh was first elucidated by phylogenetic analysis of the 56 kDa TSA gene. The Karp-related genotype was differentiated into four major clades, and Bangladeshi samples were assigned to clades 1, 2 and 4, with clades 2 and 4 being the most common. Clade 1 includes prototype strain Karp, strains JP-1 and JP-2 isolated in Japan in the 1980s, and other strains mostly distributed to East Asia. In our study, only one sample in Bangladesh was assigned to clade 1. In contrast, clades 2 and 4 are more closely related to strains in Southeast Asian countries (Taiwan, Thailand, Cambodia, etc.) as well as strains in Bangladesh. Accordingly, we suggest that clades 2 and 4 are emergent lineages which are genetically distinct from classical strains in clade 1 and which are distributed primarily to the South and Southeast Asian region.

The 56 kDa TSA is major membrane protein, and immune response to this protein is important in to protect against *O. tsutsugamushi* infection. Variable domains (VDs) in hydrophilic regions of the 56 kDa TSA have serotype/genotype-specific antigens and are associated with the induction of antibodies [7,17,18]. In the present study, amino acid sequence diversity in partial 56 kDa TSA among the Karp-related and Gilliam genotypes was analysed. Remarkably, different patterns of diversity were revealed depending on the VD. In VDI, despite few amino acid substitutions, a difference in the repeat number of the octapeptide unit was identified. A Karp-specific epitope region (amino acid 117–124) recognized by a monoclonal antibody had been mapped in VDI, overlapping with the repeat (Fig. 2) [17], and VDI was also revealed to be a highly immunogenic region inducing homotypic antibodies [18,19]. Within a same clade (1 and 2), different repeat numbers were detected, which indicated that repeat number variation might have occurred irrespective of the Karp genotypic clade. In contrast, VDII and VDIII had variable amino acid substitutions, deletions and insertions, which were generally conserved within a clade. VDII was described to be involved in antigenic domain II associated with cross-reactive antibody response [18], while information regarding antigenicity in VDIII is not available. Perhaps VDII, and possibly also VDIII, might have evolved through evasion from immune response, i.e. selection by antibodies in host. These findings on different patterns of diversity in VDs may provide a suggestion that will help design a vaccine to *O. tsutsugamushi,* especially for the Karp-related genotype.

In conclusion, the molecular epidemiologic features of *O. tsutsugamushi* were investigated in north-central Bangladesh, revealing the predominance of a Karp-related genotype associated with vast genetic diversity. Further epidemiologic study at the national level is needed to more accurately ascertain the prevalence and characteristics of this pathogen for the control of scrub typhus.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nmni.2019.100595.

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