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Detection and Molecular Characterization of *Orientia tsutsugamushi* from Suspected Scrub Typhus Patients in Mizoram, India

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ABSTRACT

Serologic and molecular tests were performed for the diagnosis and to detect *O. tsutsugamushi* genotypes that are circulating in the state of Mizoram, India. Blood samples from scrub typhus-suspected patients were collected from Synod Hospital, Durtlang, Mizoram. Weil-Felix and immunochromatographic test (ICT) were performed from the serum samples. Nested PCR (nPCR) amplification of 47kDa outer membrane protein antigen gene and 56kDa type-specific antigen gene were done from the whole blood. 141/177 (79.66%) and 134/177 (75.7%) cases showed the presence of antibody against scrub typhus by Weil-Felix and ICT assays respectively. 76/177 (42.93%) patients showed the presence of 47kDa OMP antigen gene by nPCR while 55/177 (31.07%) showed the presence of 56kDa TSA gene by nPCR. Phylogenetic analysis of 56kDa TSA gene sequence revealed that Karp-related genotype was the most common genotype in the study area followed by Kato-related genotype. In this study, a high degree of diversity of *O. tsutsugamushi* was observed similar to the observations reported from other parts of India. Nested PCR of 47kDa OMP antigen gene showed higher sensitivity as compared to nPCR amplification of 56kDa TSA gene suggesting it as the assay of choice for diagnosis of scrub typhus disease.

Introduction

Scrub typhus is an acute febrile illness. It is caused by the obligate intracellular bacterium *Orientia tsutsugamushi* and is transmitted to human by the bite of larval stages of thrombiculid mites (Seong *et al.,* 2001). Those, who inhabit regions infested with these vectors, are at high risk of acquiring scrub typhus (Tantibhedhyangkul *et al.,* 2011). It has been reported that scrub typhus disease causes illness in one million people each year,

with variable mortality rate (0–70%) and one billion people are at risk worldwide (Kelly *et al.,* 2009). Scrub typhus is endemic in geographical region known as “tsutsugamushi triangle”, which extends from Northern Japan to Eastern Russia in the north, Northern Australia in the south and Pakistan and Afghanistan in the west (Xu *et al.,* 2017). An endemic focus of scrub typhus has also been described in southern Chile (Weitzel *et al.,* 2016). A number of genotypes such as Karp, Gilliam, Kuroki, Kato, Shimokoshi, Kawasaki etc. have been recognized based on the 56 kDa type-specific antigen (TSA), which is an immunodominant outer membrane protein unique to this bacterium. Karp is the most common genotype contributing about 50% of all scrub typhus cases (Nguyen *et al.,* 2017). Genotypic characterization and detailed understanding of genetic diversity of *O. tsutsugamushi* strains in endemic regions will be essential for the development of rapid diagnostics and vaccines. Outbreaks of scrub typhus have been reported from various parts of North, South and Eastern India (Gurung *et al.,* 2013; Varghese *et al.,* 2006; Bakshi *et al.,* 2007; Kumar *et al.,* 2014). In Mizoram state, India, scrub typhus cases has also been reported from both rural and urban areas (Lalrinkima *et al.,* 2017; Lalmalsawma *et al.,* 2017; Lalthazuali *et al.,* 2020).

However, there has been no report regarding the serotypes and genotypes of *O. tsutsugamushi* from this region. Mizoram is one of the eight states of North-east India, at the extreme end of the Himalayan ranges and lies between 92°15' and 93°26'E longitude and 21°58' and 24°35'N latitude (Rosangkima *et al.,* 2018). The state has two international borders, Bangladesh in the west and Myanmar in the east. The region is influenced by monsoons, with heavy rainfall during the month of May to September. The average annual rainfall is 254 centimetres. The objective of the present study was to perform serologic and molecular tests for the diagnosis of scrub typhus and to detect *O. tsutsugamushi* serotypes that are circulating in the state of Mizoram, India.

Materials and Methods

Patient recruitment and sample collection

A prospective study of acute febrile illness was conducted during July to December, 2020 in Synod Hospital, Durtlang, Mizoram, India, and the study was approved by the Institutional Ethical Committee of Synod Hospital, Durtlang, Mizoram. 177 febrile patients who visited the hospital and 48 non-diseased control samples were included in the study. Non-diseased control samples include healthy participants from outside the hospital without any symptoms of scrub typhus disease. Patients suspected to have rickettsial illness due to the fever persisting for at least 5 days or more were enrolled in the present study. Guardian of the patients between 14 and 18 years of age were given written informed consent to participate. Cases with fever of already known causes such as malaria, typhoid, etc. were excluded in the present study. A thorough clinical history was also investigated which include questions about recent exposure to tick habitats, recent travel, similar illness in close contacts and tick bites. However, absence of these features does not rule out tick-borne illness. 3ml of venous blood was collected from each patient, 2ml in ethylene diaminetetra acetic acid (EDTA) and 1 ml in a plain tube. Serum from plain tubes were collected and subjected to Weil-Felix and immunochromatographic test (ICT).

The EDTA blood samples were also subjected to DNA extraction using QIAamp DNA Mini Kit (Qiagen, GmBh, Germany) following the manufacturer's instructions. The extracted genomic DNA was stored at -20°C for use in nested PCR amplification.

Weil-Felix test

Serological testing of OXK, OX19 and OX2 antibodies were carried out following standard protocol using PROGEN proteus antigen suspensions obtained from Tulip Diagnostics (P) Ltd., Goa, India. The serum samples were diluted and the titre values of more than 1:80 were considered positive for scrub typhus.

Rapid Diagnostic Test

InBios rapid test (SD Bioline Tsutsugamushi test, SD Diagnostics, Korea), which is an immunochromatographic strip test (ICT) designed specifically for the qualitative detection of IgM to *O. tsutsugamushi* (Wahid *et al.,* 2014). It consists of ready-to-use antigen-coated strips and reagents. The test strip was precoated with lines, one for 'T' (*O. tsutsugamushi* antibody test line), and one for 'C' (control line). The colour intensity developed on the strip is directly related to the antibody concentration.

Nested PCR amplification of 47kDa OMP antigen gene

nPCR was performed for the detection of the 47kDa OMP antigen gene using OtsuFR263 and OtsuRP1133 primers. The reaction mixture (25µl) consisted of 12.5µl of 2X MasterMix (Takara), 0.75µl each of primers (10 pmol), 9µl of nucleus free water and 2µl of DNA template. PCR reaction cycle included an initial denaturation at 94 ºC for 7 min followed by denaturation at 94ºC for 30 sec, annealing at 60ºC for 1.5 min and extension at 72ºC for 1 min.

The final extension at 72ºC takes 7 min. The second cycle of nested PCR was prepared by using 1µl of first cycle nPCR product as template and OtsuFP555 and OtsuRP771 primers producing an amplicon size of 216 bp. Other reaction components and PCR cycle conditions were similar with those in the first PCR cycle except the annealing temperature of 56°C (Srinivasan *et al.,* 2020). The PCR reaction was carried out using a ProFlex PCR System (Applied Biosystems, life technologies).

Nested PCR amplification of 56kDa TSA gene

A 56 kDa nested PCR was done in order to minimize the contamination and to get improved-sensitivity. The primers used in nested PCR were as described by Particia *et al.,* 2017 (Table 1) (Patricia *et al.,* 2017).

The first cycle of nested PCR was performed using P34 and P55 primers producing an amplicon size of 1003bp. The reaction mixture (25µl) consisted of 12.5µl of 2X MasterMix (Takara), 0.75µl each of P34 (10 pmol) and P55 (10 pmol), 9µl of Nucleus free water and 2µl of DNA template.

PCR reaction cycle included an initial denaturation at 94 ºC for 7 min followed by denaturation at 94ºC for 30 sec, annealing at 60ºC for 30 sec and extension at 72ºC for 1 min. The final extension at 72ºC takes 7 min.

The second cycle of nested PCR was prepared by using 1µl of first cycle nested PCR product as template and P10 and P11 primers producing an amplicon size of 483 bp. Other reaction components and PCR cycle conditions were similar with those in the first PCR cycle (Teppawar *et al.,* 2019). The PCR reaction was carried out using a ProFlex PCR System (Applied Biosystems, life technologies). The diagnostic assays used in the present study (Weil-Felix, ICT, nPCR of 47kDa and 56kDa genes) were assessed for sensitivity and specificity using the MedCalc statistical software program (Belgium). A total of 48 healthy, non-diseased samples were used for specificity assessment.

DNA sequencing and phylogenetic analysis

The amplified PCR products of 56kDa TSA gene of *O. tsutsugamushi* were sequenced by Sanger's dideoxy method (Sanger *et al.,* 1997) on ABI 3730XL automated sequencer (AgriGenome Labs Pvt. Ltd., SmartCity Kochi, Kerala, India). Nucleotide sequences obtained from the present study were deposited in the GenBank data library under accession numbers MW620836 to MW620866. Sequences were edited using BioEdit Sequence alignment editor and subjected to phylogenetic tree construction. A phylogenetic dendrogram of the partial 56kDa protein genes in the present study as well as those obtained from the GenBank database was constructed by the maximum likelihood method using MEGA7 software (Kumar *et al.,* 2016). Sequence identity among 56kDa TSA genes was calculated by the Sequence Identity and Similarity (SIAS) programme (http://imed.med.ucm.es/Tools/sias.html).

Results and Discussion

A total of 177 scrub typhus-suspected patients were included in this study. The median age of the patients was 42.5 years (ranging from 15 to 86 years). 141/177 (79.66%) cases showed scrub typhus positive by Weil-Felix test, of which 53.1% were female and 26.5% were male. Immunochromatographic test showed 134/177 (75.7%) scrub typhus positive with 32.7% female and 42.9% male. Nested PCR of the 47kDa OMP antigen gene showed 76/177 (42.93%) positive with 20.3% female and 22.6% male. Nested PCR of 56kDa TSA gene also showed 55/177 (31.07%) positive with 16.4% female and 14.7% male (Table 2; Figure 1). The common symptoms noted among the patients included fever, vomiting, abdominal pain, myaglia, chills, headache and jaundice. Among the four diagnostic assays used for scrub typhus disease in the present study, Weil-Felix test showed the highest sensitivity of 79.66% (95% CI, 72.9 to 85.3) but lowest specificity of 87.41% (95% CI, 75.5 to 94.7). Nested PCR of 47kDa OMP antigen gene showed higher sensitivity of 42.94% (95% CI, 35.5 to 50.5) as compared to nPCR of 56kDa TSA gene showing sensitivity of 31.07% (95% CI, 24.3 to 38.4).

Neither 47kDa nor 56kDa nPCR showed positive for non-diseased samples, and also the primers used were species-specific primers designed for *O. tsutsugamushi,* thereby, revealing perfect specificity (100%) of both assays (Table 3).

The 56kDa TSA gene products (483 bp) were sequenced for randomly selected 37 positive samples. A phylogenetic tree was constructed for these 37 samples together with 40 reference strains (Figure 2). Phylogenetic and sequence analysis revealed that Karp-like strains predominated. Thirty one samples (83.7%) out of 37 samples analyzed in the present study clustered within the Karp-related genotype. Four samples (10.8%) clustered within the Kato-related genotype, while only one sample each clustered within the Gilliam and TA763-related genotypes. Karp-related genotypes were phylogenetically differentiated into four clades (clade 1, 2, 3 and 4). Seventeen samples were assigned into clade 1, three samples into clade 2, two samples into clade 3 and nine samples into clade 4. They were clustered with strains from Taiwan, Korea, Thailand, India, China, Japan and Vietnam. Within the partial 56 kDa TSA gene, Karp-related genotype from different clades showed sequence identity of 89.2% - 99.6%, while the sequence identity between Karp-related and Gilliam genotypes was 62% - 91% (Table 4). Scrub typhus is prevalent in Asia-Pacific area especially in rural areas of South-East Asia (Rapmund, 1984). However, it is difficult to diagnose due to its non-specific presentations, and lack of relevant laboratory tests (Sinha *et al.,* 2014).

In India, scrub typhus has been reported from many states accounting for up to 50% of undifferentiated febrile illnesses during cooler season (Mathai *et al.,* 2003; Abhilash *et al.,* 2015). Scrub typhus responds well to proper antibiotic treatment. However, delayed diagnosis may cause fatal complications (Silpapojakul *et al.,* 1991). Therefore, a rapid diagnosis of scrub typhus is important to get successful treatment.

Weil–Felix is the oldest test and is easy to perform but lacks sensitivity. Due to low antibody levels during the early stages of infection, serological tests may fail to diagnose scrub typhus (Huber *et al.,* 1012). This may reflects the low sensitivity of Weil-Felix test in some cases. However, during the later stages of infection, it showed higher sensitivity as observed in the present study. Therefore, it is recommended that the rapid card test should be interpreted in combination with other diagnostic assays and clinical findings (Wahid *et al.,* 2014). In the present study, Weil-Felix and ICT tests were performed as a screening test followed by nPCR targeting 47kDa OMP antigen gene and 56kDa TSA gene as confirmatory tests. The sensitivity (79%) and specificity (87%) of Weil-Felix test in the result of present study are slightly higher than the range of sensitivity and specificity given by the manufacturer (70% each). However, the sensitivity of ICT observed in the present study (75%) was quite lower than the range given by the manufacturer (99%) while, specificity (93%) was close to manufacturer range (96%). PCR-based molecular diagnosis targeting different genes have been used in scrub typhus cases. Although the sensitivity of these molecular methods for detection of the target gene is low, they have a very high specificity of up to 100%. The lower sensitivity of these PCR-based molecular methods reported from earlier studies (Kannan *et al.,* 2020) and in the present study could probably be due to the genetic variability associated with the target genes.

Table.1 Details of primer sets used in the present study.

Gene detected	Primer details		Product size
47kDa nPCR	(Cycle 1) OtsuFP263 (Forward) OtsuRP1133 (Reverse)	5'-GTGCTAAGAAARGATGATACTTC-3' 5'-ACATTTAACATACCACGACGAAT-3'	216 bp
	(Cycle 2) OtsuFP555 (Forward) OtsuRP771 (Reverse)	5'-TCCTTTCGGTTTAAGAGGAACA-3' 5'-GCATTCAACTGCTTCAAGTACA-3'	
56kDa nPCR	(Cycle 1) P34 (Forward) P55 (Reverse)	5'-TCAAGCTTATTGCTAGTGCAATGTCTGC-3' 5'-AGGGATCCCTGCTGCTGTGCTTGCTGCG-3'	483 bp
	(Cycle 2) P10 (Forward) P11 (Reverse)	5'-GATCAAGCTTCCTCAGCCTACTATAATGCC-3' 5'-CTAGGGATCCCGACAGATGCACTATTAGGC-3'	

Table.2 Number of positive by different diagnostic assays per a total of 177 cases.

Weil Felix		ICT		nPCR (47kDa)		nPCR (56kDa)	
Female	Male	Female	Male	Female	Male	Female	Male
94	47	58	76	36	40	29	26
53.1%	26.5%	32.7%	42.9%	20.3%	22.6%	16.4%	14.7%

Table.3 Sensitivity and specificity of different diagnostic assays.

Diagnostic assays	Percent sensitivity		Percent specificity	
	Value	95% CI	Value	95% CI
Weil Felix	79.66%	72.97% to 85.33%	87.27%	75.52% to 94.73%
ICT	75.71%	68.70% to 81.83%	93.75%	82.80% to 98.69%
47kDa nPCR	42.94%	35.54% to 50.58%	100%	92.60% to 100%
56kDa nPCR	31.07%	24.34% to 38.45%	100%	92.60% to 100%

Table.4 DNA sequence identity (%) of partial 56kDa type-specific antigen gene among the present study samples with Karp and Gilliam genotypes.

Strain/sample	Karp	KP18	KP21	KP71	KP33	NT83	Gilliam
KP18	98.9						
KP21	94.4	87.5					
KP71	92.9	93.4	90.5				
KP33	94.7	89.2	93.4	95.8			
NT83	99.6	99.0	94.6	92.9	94.9		
Gilliam	90.9	66.9	76.7	72.3	77.2	76.7	
NT19	77.5	62.2	71.1	66.5	76.2	75.7	93.6

Fig.1 Bar diagram showing the percentage positivity of different diagnostic assays on scrub typhus suspected cases.

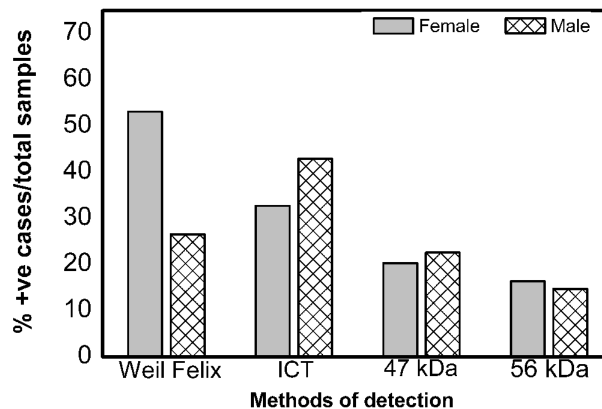
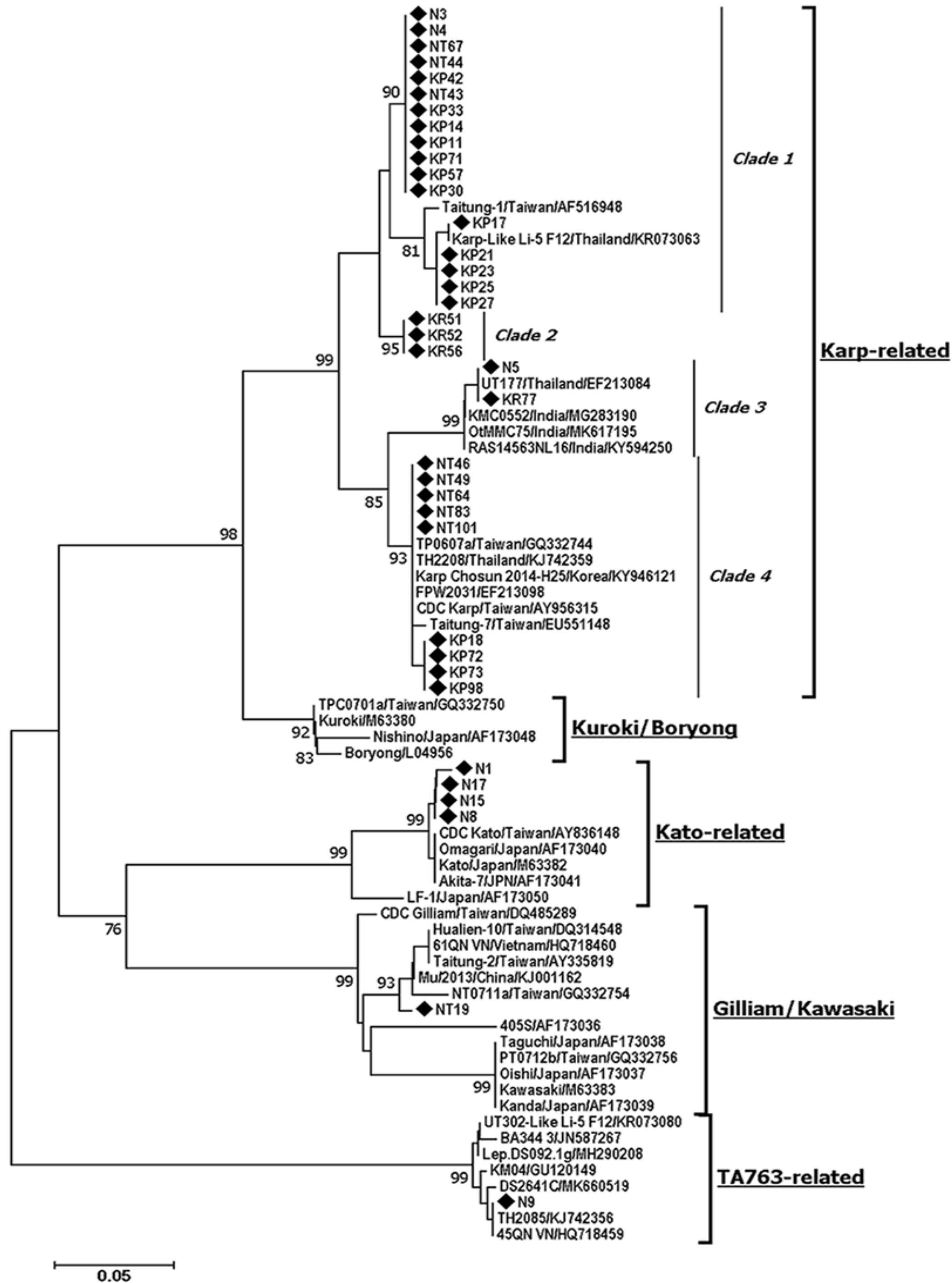


Fig.2 Phylogenetic dendrogram of partial 56kDa TSA gene sequences generated for 77 samples (37 from the present study; 40 from reference strains). The tree was constructed using the neighbour-joining method in MEGA7 software. Tree was statistically supported by bootstrapping with 1000 replicates. Percentage bootstrap support values greater than 75 were shown.



There are a large number of studies documenting the usefulness of 47kDa for diagnosing scrub typhus (Huber *et al.,* 2012; Kim *et al.,* 2011; Jiang *et al.,* 2004). It is as sensitive as nPCR of other genes. The sensitivity ranges from 40% to 80%, with specificity of up to 100% (Peter *et al.,* 2015; Watthanaworawit *et al.,* 2013; Janardhanan *et al.,* 2014). The result of present study presented the diagnostic efficiency of Weil-Felix, ICT and nPCR of 47kDa OMP and 56kDa TSA genes. Our study revealed highest sensitivity with Weil-Felix assay followed by ICT. However, these two assays exhibited comparatively lower specificity against molecular methods. Nested PCR of 47kDa OMP gene showed higher sensitivity (42.94%) as compared to nPCR of 56kDa TSA gene (31.07%). The lower sensitivity of nPCR of 56kDa TSA gene may be due to low-copy number of target DNA or excess number of host DNA.

More than 20 prototype strains of *Orientia tsutsugamushi* have been documented so far (Kelly *et al.,* 2009). However, in India, only a few studies have been conducted from the north, south and north-east India regarding the genetic diversity of circulating strains (Varghese *et al.,* 2013; Bora *et al.,* 2018). In the present study we have documented the genetic diversity of *O. tsutsugamushi* in Mizoram state, India. Phylogenetic analysis of 56kDa TSA gene in the present study revealed the presence of Karp-related, Kato-related, Gilliam-related and TA763-related strains in the study area. Karp-like strains predominated followed by Kato-related strains, while Gilliam and TA763-related strains were detected only on one sample each. In India, the presence of Gilliam and Karp prototypes along with other genotypes closely related to Kuroki, Boryong, Chuto and Kato have been reported (Patricia *et al.,* 2017; Kumar and Beena, 2017). Kato-like strain has also been reported to be very common across the country. In the present study area also Karp and Kato-related strains were comparatively more common than the other strains. The limitation of this study is that the present study was done over a short period of time and included only participants from a single Hospital in the state.

The most prevalent genotype identified in the present study was those in the Karp-related strain. However, considering the higher strain variation across the country, further prospective studies in this area will be required to identify the prevalent strains as well as antigenic variations. As compared to nPCR of 56kDa TSA gene, nPCR of 47kDa OMP antigen is more sensitive suggesting it as the assay of choice for diagnosis of scrub typhus disease.

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