



Molecular characterization and evolutionary analysis of *Orientia tsutsugamushi* in eastern Indian population

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Abstract

Scrub typhus is a bacterial zoonotic acute febrile illness (AFI) caused by the obligate intracellular bacterium *Orientia tsutsugamushi*, which is an antigenically diverse strain frequently observed in the tropical region of Southeast Asian countries. The recent investigation was conducted to delineate the genotype identification of *Orientia tsutsugamushi* predominating in the eastern zone of India such as Odisha to decipher its strain type, and evaluate its diversity as well as evolutionary pattern based on the nucleotide analysis of the immune dominant 56 KDa gene. During this study, we have investigated 100 clinical samples (2014–2018), out of which 28 were positive for scrub typhus followed by its molecular characterization and phylogenetic analysis utilizing 56 KDa partial genes. Population genetic parameters showed the presence of 287 polymorphic sites within the analyzed 56 KDa gene. The gene diversity (H_d) and sequence diversity (π) was estimated 0.638 and 0.280, respectively. Selection pressure analysis ($\theta = dN/dS$) having the value 0.222 suggests that the gene lied under purifying selection. The present study suggested a high rate of genetic diversity within the isolates. This research study sheds light on the hereditary and evolutionary relationships of *Orientia* strains found in the eastern Indian population. Understanding regional genetic variation is critical for vaccine development and sero-diagnostics methods. A significant level of genetic variability was observed during this study. This information has a way to understand more about antigen diversity that leads to develop an effective vaccine candidate for this pathogen.

Keyword Scrub typhus · *Orientia tsutsugamushi* · Vector-borne disease · Genetic diversity · Phylogenetic tree

Introduction

Scrub typhus is a vector-borne zoonotic bacterial infection caused by bacteria, *Orientia tsutsugamushi* (OT). It is a Gram-negative obligate intracellular parasite that belongs to genus *Orientia*, family *Rickettsiaceae* with significant genetic differences in peptidoglycan and lipopolysaccharide (LPS) compared with *Rickettsia* genus (Hayashi 1920; Ogata 1931; Tamura et al. 1995). The pathogen has worldwide

distribution and promotes acute febrile illness (AFI) (Khan et al. 2017). The disease manifested by high temperature for 14 days lacks other clinical symptoms. Latter the infection leads to lymphadenopathy, rash, interstitial pneumonitis, meningitis, and myocarditis, and causes serious problems in vital organs like the lungs, liver, kidneys, and central nervous system (Jeung et al. 2016). Therefore, this disease is clinically indistinguishable from infection-causing acute fever, such as malaria, dengue, other rickettsioses, and leptospirosis (Kundavaram et al. 2013; Acestor et al. 2012). The mortality rate differed and can be reached up to 50% (Hu et al. 2015). OT is introduced into humans through trombiculid mites bite, those belonging to the genus *Lep-totrombidium*. People who share the geographical regions with these vectors are at high risk of infection (Wardrop et al. 2013). Vectors usually prefer temperate and rainfall climates for their proliferation and spread.

The genome size of the bacterium varies from 1.93 to 2.47 Mb with a low GC content of 30–31%. The genome consists of 2086–2709 genes; those were enriched with

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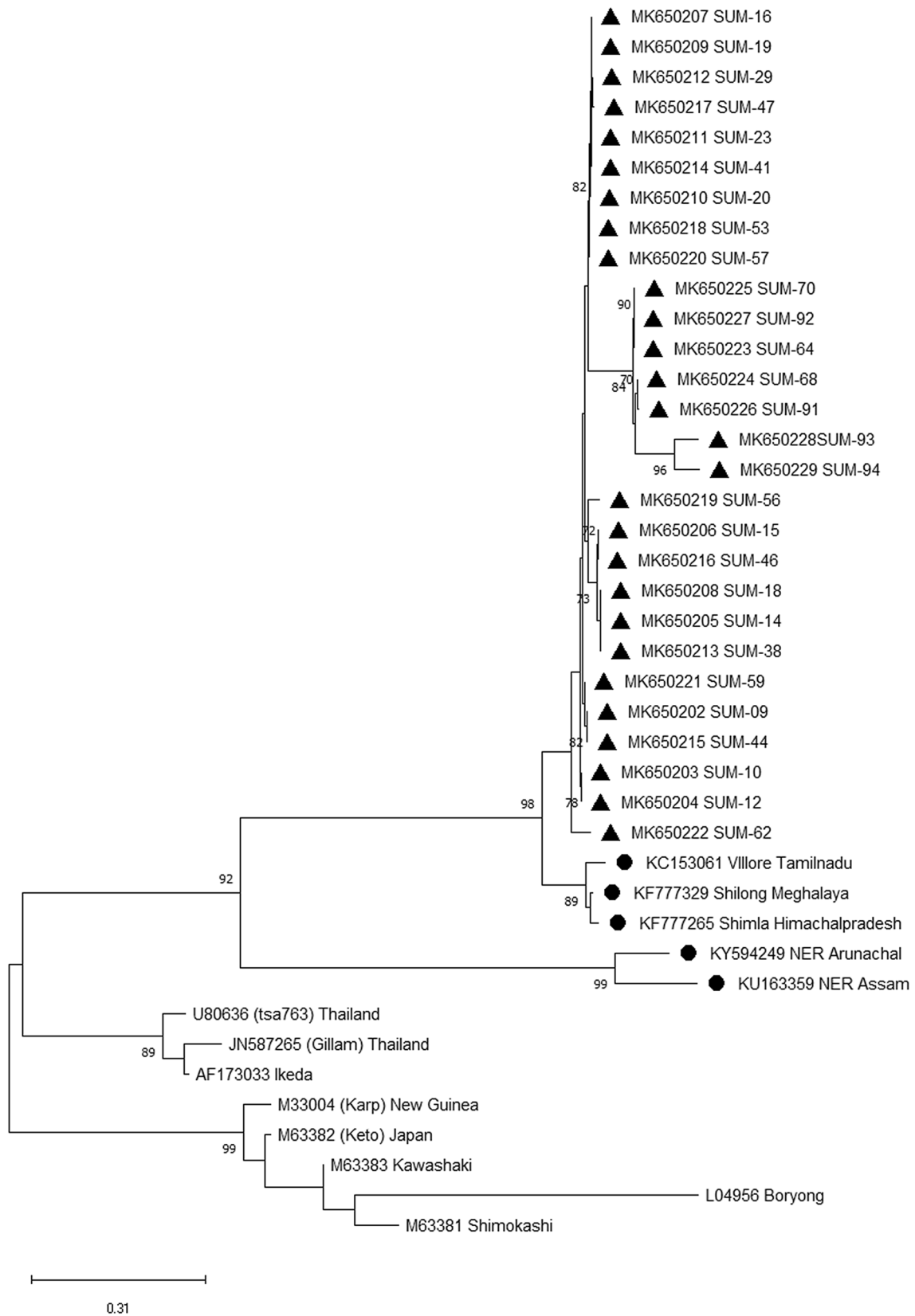


Fig. 1 Phylogenetic analysis utilizing 56-kDa partial gene. Phylogenetic relationships of *Orientia tsutsugamushi* detected in human patients with scrub typhus in eastern Indian population. Relationships were determined based on the partial 56-kDa type-specific antigen gene by the minimum-evolution method with the Kimura 2-parameter distance model. Bootstrap values >50% are shown at the branches. Location and GenBank accession numbers are indicated for each sequence. A solid triangle indicates sequences determined in this study

repeat, which contributes 37.1% of the total genome (Batty et al. 2018). Recently, observed that OT interferes with $\alpha 5\beta 1$ integrin-mediated signaling pathway to seize the replication of HeLa cells (Cho et al. 2010). Some reports suggested that during phagocytosis by L929 cells, OT exits the phagosome and goes into the cytoplasm (Chu et al. 2006). OT also regulates the clathrin-mediated endocytosis signaling pathway to infect endothelial and fibroblast cell lines. This leads to affect the organ like the lungs, liver, kidneys, and central nervous system (Jeung et al. 2016). For strain identification and genotype demarcation, the groES and groEL genes, the membrane proteins 47 kDa, and 56 kDa genes were extensively used worldwide due to their high diversity (Arai et al. 2013; Jiang et al. 2013; Lu et al. 2010). Due to the high immunogenic response in human and animal models, the 56 kDa and 47 kDa genes are used for vaccines. However, 120 antigenically different strains have been described which are further classified as Karp, Gilliam, and Kato (Kelly et al. 2002; Varghese et al. 2015). The antigenic diversity acts as an obstacle to develop an effective vaccine candidate. The sequence of a highly diverse immune dominant gene such as 56KDa, unique to OT, has become an important tool for strain demarcation (Blacksell et al. 2008).

The disease has a worldwide distribution including Japan, China, Korea, Philippines, Australia, Chile, India, Pakistan, Taiwan, and Afghanistan (Xu et al. 2017; Kuo et al. 2012). Reinfections were common due to the diversity of strains (Smadel et al. 1949). Scrub typhus has recently been observed in India and is now thought to be a major cause of severe febrile sickness. Limited information is available throughout India concerning its strain types (Mahajan et al. 2006; Varghese et al. 2015). Although a few reports suggested the endemicity of this disease within the Northeast region such as Assam and Meghalaya having Karp-like strains and Gilliam-like strains, respectively (Khan et al. 2017; Varghese et al. 2015). Still, continuous surveillance is required to get a clear picture to reveal the prevalence of genotype circulating within India. Thus, in the present investigation, the sequence information of the immunological dominant 56 KDa gene was utilized to define the strains of OT circulating in Odisha, a state of eastern India, and deciphered its genotype followed by estimation of genetic diversity and evolutionary trend.

Material and methods

Clinical sample collection from outbreak areas

Blood samples were collected from suspected humans from 28 outbreaks that occurred in different locations of Odisha during the years 2014–2018. The primary symptoms of infection comprised of headache, altered mental changes, vomiting, unconsciousness, nausea. These clinical samples were kept in an Eppendorf tube treated with aseptic phosphate-buffered saline (PBS) having pH 7.2, treated with antimicrobial reagents, and kept at -20°C for further processes.

DNA isolation

DNA from human blood was obtained utilizing the HipurA DNA blood kit (Himedia, India). One hundred μl of blood samples were processed to eliminate DNA according to the manufacturer's protocol. DNA was extracted utilizing 100 μl of the elution buffer provided by the kit followed by its transfer into a 0.6 ml cryovial and stored at -20°C for future use (Fig. 1).

PCR amplification

Nested PCR was obtained following the method used by Furuya et al. 1993 with some modifications (Furuya et al. 1993). PCR primer F1-5'-TCA AGC TTA TTG CTA GTG CAA TGT CTG C-3'; R1-5'-AGG GAT CCC TGC TGC TGT GCT TGC TGC G-3' and F2-5'-GAT CAA GCT TCC TCA GCC TAC TAT AAT GCC-3'; R2-5'-CTA GGG ATC CCG ACA GAT GCA CTA TTA GGC-3' for the nested PCR to get the 56-kDa amplicons (Lee et al. 2011). The PCR master mix (25 μl) was composed of 5 pmol of each primer, 2 mM dNTPs in $1\times$ buffer supplemented with 2.5 U Taq polymerase according to the supplier's recommendations (NEB). The PCR reaction conditions were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 50 s, annealing at 55°C for 60 s, extension at 72°C for 90 s, and a final extension at 72°C for 7 min. The PCR amplified products (10 μl) were stacked into a 1% agarose gel. Furthermore, these amplicons were gel-purified utilizing a QIAquick Gel Extraction Kit (QIAGEN) and sequenced commercially.

Construction of phylogenetic tree and evaluation of population genetics parameters

The nucleotide sequences of the 56-kDa gene were verified and aligned by the Bioedit software version 7.0.5.3 to get a consensus sequence. MEGA 7 was utilized to construct a

phylogenetic tree through the minimum-evolution method with a bootstrap value of 1000 replicates (Saitou and Nei 1987). The polymorphic sites, haplotype diversity (Hd), nucleotide diversity (π), selection pressure (dn/ds), and Tajima D of the population were calculated using DnaSP version 5.10.01 (Librado and Rozas 2009).

Results

Phylogenetic analysis

Primers targeting the 56 KDa gene amplified in 28 clinical samples having fragments size 464 bp. The respective sequences were submitted to the GenBank to get the accession number MK650201–MK650229 (Table 1). A phylogenetic tree was constructed utilizing the sequence submitted during this study along with all 13 available sequences retrieved from GenBank. The nucleic acid and amino acid

sequence of the present isolates revealed 99.0–100% and 98.2–100% similarities, respectively. The phylogenetic tree consists of five groups, from which group I and group II mostly have Indian isolates. All isolates of the present study exhibited in one group having a close relation with Meghalaya isolate.

Population genetic analysis

Population genetic parameters showed the presence of 287 polymorphic sites within the analyzed gene. The Haplotype diversity (Hd) and nucleotide diversity (π) were observed 0.638 and 0.280, respectively. Selection pressure analysis ($\theta = dN/dS$) having the value 0.222 suggests that the gene lied under purifying selection. This selection of virulence genes could enhance the mutation rate, which induces a strong host immune response. Tajima's D test otherwise known as the neutrality test of this gene resulted in a value

Table 1 Details of *Orientia tsutsugamushi* infected children and the location of the samples collected in this study

Sr no.	Disease	Host species	Geographic area of distribution	Coordinates	Year of outbreak	Accession no.
1	Scrub typhus	Human	Nayagarh	20.1231° N, 85.1038° E	2014	MK650202
2	Scrub typhus	Human	Puri	19.8135° N, 85.8312° E	2014	MK650203
3	Scrub typhus	Human	Khurda	20.1301° N, 85.4788° E	2014	MK650204
4	Scrub typhus	Human	Nayagarh	20.1231° N, 85.1038° E	2015	MK650205
5	Scrub typhus	Human	Khurda	20.1301° N, 85.4788° E	2015	MK650206
6	Scrub typhus	Human	Nayagarh	20.1231° N, 85.1038° E	2015	MK650207
7	Scrub typhus	Human	Mayurbhanj	21.5140° N, 86.5325° E	2015	MK650208
8	Scrub typhus	Human	Khurda	20.1301° N, 85.4788° E	2015	MK650209
9	Scrub typhus	Human	Dhenkanal	20.6505° N, 85.5981° E	2015	MK650210
10	Scrub typhus	Human	Khurda	20.1301° N, 85.4788° E	2016	MK650211
11	Scrub typhus	Human	Nayagarh	20.1231° N, 85.1038° E	2016	MK650212
12	Scrub typhus	Human	Koraput	18.8135° N, 82.7123° E	2016	MK650213
13	Scrub typhus	Human	Nayagarh	20.1231° N, 85.1038° E	2016	MK650214
14	Scrub typhus	Human	Khurda	20.1301° N, 85.4788° E	2016	MK650215
15	Scrub typhus	Human	Nayagarh	20.1231° N, 85.1038° E	2016	MK650216
16	Scrub typhus	Human	Khandagiri	20.1301° N, 85.4788° E	2017	MK650217
17	Scrub typhus	Human	Cuttack	20.4625° N, 85.8830° E	2017	MK650218
18	Scrub typhus	Human	Khurda	20.1301° N, 85.4788° E	2017	MK650219
19	Scrub typhus	Human	Khurda	20.1301° N, 85.4788° E	2017	MK650220
20	Scrub typhus	Human	Nayagarh	20.1231° N, 85.1038° E	2018	MK650221
21	Scrub typhus	Human	Puri	19.8135° N, 85.8312° E	2018	MK650222
22	Scrub typhus	Human	Khurda	20.1301° N, 85.4788° E	2018	MK650223
23	Scrub typhus	Human	Ganjam	19.5860° N, 84.6897° E	2018	MK650224
24	Scrub typhus	Human	Ganjam	19.5860° N, 84.6897° E	2018	MK650225
25	Scrub typhus	Human	Khurda	20.1301° N, 85.4788° E	2018	MK650226
26	Scrub typhus	Human	Puri	19.8135° N, 85.8312° E	2018	MK650227
27	Scrub typhus	Human	Nayagarh	20.1231° N, 85.1038° E	2018	MK650228
28	Scrub typhus	Human	Khurda	20.1301° N, 85.4788° E	2018	MK650229

of -1.14586 . The negative value revealed that, this pathogen might adapt to evolutionary expansion.

Discussion

Scrub typhus is commonly referred to as an acute febrile illness observed in the rural part of South Asia. The disease is manifested by the presence of fever, myalgia, chills, headache, fatigue, loss of appetite, and gastrointestinal symptoms such as nausea, vomiting, cough, diarrhea, and skin rash. Other symptoms include obstetric complications in pregnant women which lead to poor pregnancy outcomes. Demographic data confirmed that it can be contracted by all age groups and farmers were the most affected profession. The causative agent of this infection is *Orientia tsutsugamushi*, which infects endothelial cells macrophages, monocytes, dendritic cells, or cardiac myocytes which leads to a variety of complications in the liver and kidneys (Paris et al. 2015).

In the present study, the common symptoms of positive patients had fever, headache, myalgia, conjunctivitis, and rash. A few patients also suffered from cough, vomiting, nausea, abdominal pain, lymphadenopathy, hepatomegaly, conjunctivitis, rash, and splenomegaly. The phylogenetic analysis indicates the presence of five groups and the current isolates belong to the Kato genotype. However, several studies in India suggested the presence of Kato genotype, and have sequence similarity with OT isolates from Korea, Taiwan, Vietnam, and Cambodia (Fournier et al. 2008; Duong et al. 2013). In India, the disease has been frequently reported from southern, northern, and northeast regions of the country (Varghese et al. 2015; Khan et al. 2017). The partial sequences of 56 kDa gene revealed the prevalence of Kato strains in South India; Karp-like strains in North India; Gilliam and Karp strain in North-eastern (NE) India (Varghese et al. 2015; Khan et al. 2017). The NE region acts as a gateway for trade exchange between India and South Asian countries. That might be responsible for the introduction of the new strain into the mainland of India. The present study suggested a high rate of genetic diversity within the isolates. The previous study suggested the presence of an elevated rate of recombination, genome duplication, and horizontal gene transfer to maintain the genetic diversity of OT isolates in Thailand (Fournier et al. 2008), Cambodia (Duong et al. 2013), and China (Long et al. 2020). These recombination events are usually responsible for substantial genetic polymorphism (Yang et al. 2012), and variable antigenicity (James et al. 2016). Along with that cause, geographical expansion of scrub typhus may lead to an increase the diversity.

The higher genetic diversity hinders to development of an effective universal vaccine (Ni et al. 2005). Continuous emergence and re-emergence of this disease indicate its

pandemicity (Ranjan and Prakash 2018). However, changes in human behavior, unplanned urbanization, and deforestation lead to the displacement of vectors as well rodents from one place to another (Yang et al. 2014; Park et al. 2015); that may introduce the pathogen in a new geographical area and spread the disease. The available vaccine of scrub typhus vaccine utilized the 56 kDa immune dominant protein, which provides homologous protection, but is unable to produce heterologous immunity (Chattopadhyay and Richards 2007). Our study might be helpful to develop an effective vaccine or antiviral drugs for disease eradication. During genetic recombination, the nonessential genes may be eliminated, or host genes may be inserted to generate new strains in the population. Some recent transcriptomic analysis revealed that the bacterial genotype plays a major role in the differential expression of immune regulatory genes (Mika-Gospodorz et al. 2020; Salje 2021). Therefore, one can assume that the introduction new genotype mimics the host factor and regulate the virulence of the respective pathogen. Due to the absence of enough genomic resources as well as complete genome sequence from India, it is impossible to decipher more about this pathogen biology. Therefore, our next objective would be revealing the complete genome information of the respective strain from India followed by the development of an appropriate vaccine for preventive interventions of this disease.

Conclusion

The present investigation suggests the surveillance followed by a molecular approach to decipher genetic heterogeneity exhibited within *Orientia* strains found in the eastern Indian population. A significant level of genetic variability was observed during this study. This information has a way to understand more about antigen diversity that leads to developing an effective vaccine candidate for this pathogen.

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Author contributions SKS designed the Research, editing, and writing throughout the manuscript. BPS, SP, and RS analyzed the results of the research study, and wrote and edited the manuscript. All authors corrected the manuscript for the final draft.

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Availability of data and materials The partial nucleotide sequences of the 56-kDa gene of the present study can be obtained from the GenBank repository (<http://www.ncbi.nlm.nih.gov/genbank>) with the accession number MK650202-MK650229, respectively.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they do not have any conflict of interest.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent to publication Not applicable.

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