International Journal of Infectious Diseases 84 (2019) 131–135

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Genetic diversity of *Orientia tsutsugamushi* strains from patients in north India

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ARTICLE INFO

Article history:
Received 6 January 2019
Received in revised form 25 April 2019
Accepted 26 April 2019
Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:
Scrub typhus
Orientia tsutsugamushi
Eschar
North India

ABSTRACT

Background: Scrub typhus has emerged as a major cause of acute febrile illness in India in recent years. The causative agent, *Orientia tsutsugamushi* has more than 20 prototype strains due to a variable 56-kDa outer membrane protein. It is crucial to know the prevailing types in India for the success of diagnostic immunoassays and prospective vaccine candidates. In north India, the principal types circulating are largely unknown. Our tertiary care hospital caters to a large area of north India (around 7 states and one union territory). Therefore, the current study was planned to identify the genotypes of *O. tsutsugamushi* strains circulating in this wide area of north India.

Materials and methods: Adults and children presenting with suspected scrub typhus between July 2013 and December 2013 were included in this study. DNA was extracted from whole blood and a nested PCR was used to amplify a 483-bp region of the 56-kDa antigen gene of *O. tsutsugamushi*. The PCR products were purified and DNA sequencing was performed and aligned using the CLUSTAL_X2 program. A phylogenetic tree was constructed using neighbour- joining algorithms and analysed using the sequences obtained in this study and those obtained from the GenBank database.

Results: A total of 34 samples were positive for PCR. The amplicons were sequenced and analyzed. Karp-like strains predominated in all states studied (64.7%) followed by Gilliam-like (26.47 %) and 8.82% similar to Hualien 1 or S072. We did not find any Kato or Kawasaki-like strain. Karp like strains showed >99% similarity to TH2033, TH2191, TH2208, Xinjiang & Neimeng strains and Gilliam-like strains showed >99% similarity to Clone ISS -11.

Conclusion: Orientia tsutsugamushi shows a great diversity in its strains over a large geographical area of north India. This has implications in the production of both diagnostic assays and vaccine for scrub typhus.

Introduction

Scrub typhus has emerged as a major cause of acute febrile illness (AFI) in India in recent years (Sethi et al., 2014; Khan et al., 2017; Abhilash et al., 2016; Jain et al., 2018a). AFI refers to fever with oral temperature more than 101 °F for less than 14 days duration without localizing clinical features. The clinical features can vary from mild symptoms to severe life-threatening illness (Sharma et al., 2016; Varghese et al., 2014). The causative agent, Orientia tsutsugamushi has several antigenic types because of the variation in the 56-kDa protein on the outer membrane. There are at least twenty antigenically different strains, three major prototypes being Gilliam, Karp and Kato (Tamura et al., 1984). It is crucial to know the prevailing antigenic types in India as the success of diagnostic immunoassays and prospective vaccine candidates is dependent on this knowledge. There are only a few previous studies on the principal antigenic types circulating in our huge country (Varghese et al., 2015; Bakshi et al., 2007; Mahajan et al., 2006; Koraluru et al., 2016). Our tertiary care hospital caters to around eight states of north India. Most of the patients come from neighbouring states like Punjab, Haryana, Himachal Pradesh

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https://doi.org/10.1016/j.ijid.2019.04.030

and many districts of Uttar Pradesh and Jammu and Kashmir. Therefore, the current study is planned to identify the genotypes of O. tsutsugamushi detected in patients with scrub typhus from this huge region of north India.

Methods

All patients with acute undiagnosed febrile illness presenting in outpatient or inpatient services of PGIMER Chandigarh between July 2013 and December 2013 were included in this study. The study was approved by the institutes' ethical committee. A detailed risk factor and clinical history was taken and a thorough examination of patients was made to look for eschar and rash. Whole blood and serum was collected from patients for PCR and IgM ELISA respectively. Serum samples of these patients were subjected to testing for specific IgM antibodies against O. tsutsugamushi using a commercial ELISA kit (InBios International Inc. USA) which uses O. tsutsugamushi derived recombinant antigen mix. The test was performed as per manufacturer's instructions.

DNA from 2 ml of whole blood samples was extracted using a DNA extraction kit. Positive control (*O. tsutsugamushi* Karp strain DNA) was donated by Dr Allen Richards, Naval Medical Research Centre, USA. The nucleotide primers were taken from gene encoding for the 56-kDa antigen of the Gilliam strain of *O. tsutsugamushi* (Furuya et al., 1991). A nested PCR was used to amplify a 483-bp region of the 56-kDa antigen gene.

The PCR products were purified and DNA sequencing was performed and aligned using the CLUSTAL_X2 program. A phylogenetic tree was constructed using neighbour-joining algorithms and analyzed using the sequences obtained in this study and those obtained from the GenBank database.

Results

A total of 293 patients with history of acute febrile illness during this study period were included. Out of 293 (146 males and 147 females) patients, 215 were adults and 78 were children. The age of the patients ranged from 0.1 to 82 years with median age 26 years. The mean age of 37.2 years was found in adults and 7.8 years in children. The occupation history was available for 101 cases, of which 44(43.5%) were farmers while 41(40.5%) were housewives. The patients hailed from 5 states (Haryana, Punjab, Himachal Pradesh, Uttar Pradesh, Uttarakhand) and one union teriterry (Chandigarh). The majority of them were from Haryana (32%) followed by Punjab (24%) and Himachal Pradesh (20.5%).

High-grade fever associated with chills and rigor (48.12%) was the most common presenting symptom in 82.25% (median duration of fever at presentation was 10 days). Breathlessness was the presenting feature in 37%, abdominal pain in 26%, jaundice in 25%, rashes in 16%, renal failure in 10%, diarrhea in 9%, and seizures in 7%. A pathognomonic eschar was found in 14% of the patients. ELISA was positive in 228 (78%), PCR in 34 (11.6%) patients, and both were positive in 29(9.89%) patients. (In four patients ELISA was not performed due to unavailability of serum sample.) The geographical distribution of the PCR positive cases represented in Figure 1. Thirty four of these PCR products were purified and DNA sequencing was performed and aligned using the

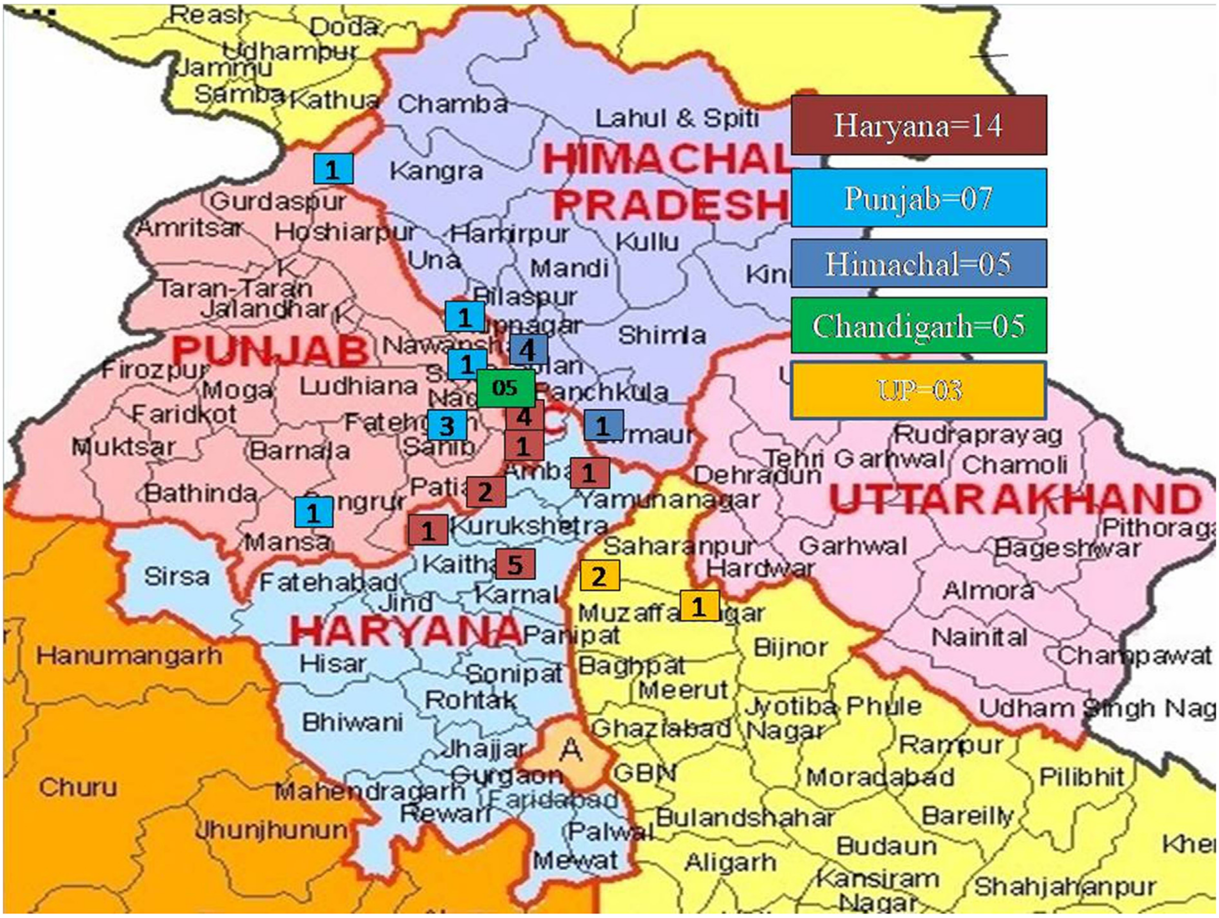

Figure 1. Geographical distribution of scrub typhus PCR positive cases in our study.

CLUSTAL_X2 program. A phylogenetic tree was constructed using neighbour- joining algorithms and analysed using the sequences obtained in this study and those obtained from the GenBank databases (Figure 2) (Saitou and Nei, 1987; Tamura et al., 2004). These sequences were registered in the GenBank (accession numbers received were KT727932-KT727943, KT735173-KT735176, KT957867-KT957881, KT960999, KT961000 and KT967113). Karp-like (64.7%) strains predominated, followed by Gilliam-like (26.47%) strains and those similar to Hualien1 or S072 (8.82%). We did not find any Kato-like strains or Kawasaki-like strains. Karp like strains showed >99% similarity to TH2033, TH2191, TH2208, Xinjiang & Neimeng strains and Gilliam- like strains showed >99% similarity to Clone ISS-11. A second dendrogram was constructed using strains from Indian studies (Figure 3). The evolutionary divergence between our sequences and other prototype strains has been shown in Table 1 (Furuya et al., 1991). One sequence, PGI34CHD with accession number KT735173 showed significant genetic distance (1.455) from Vellore strain and minimum distance (0.246) from Boryong strain. The sequence PGI34CHD showed some distance from strain Karp (0.274) and other strains from India. In homology studies of the

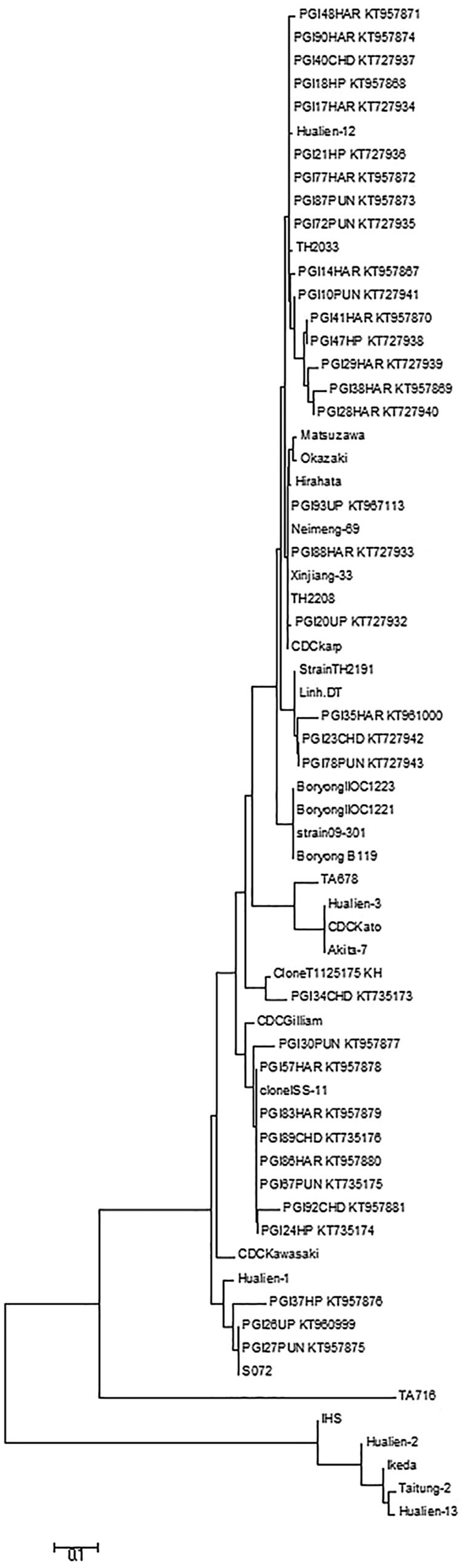

Figure 2. Phylogenetic analysis of scrub typhus strains by the Neighbor-Joining method, conducted in MEGA6.

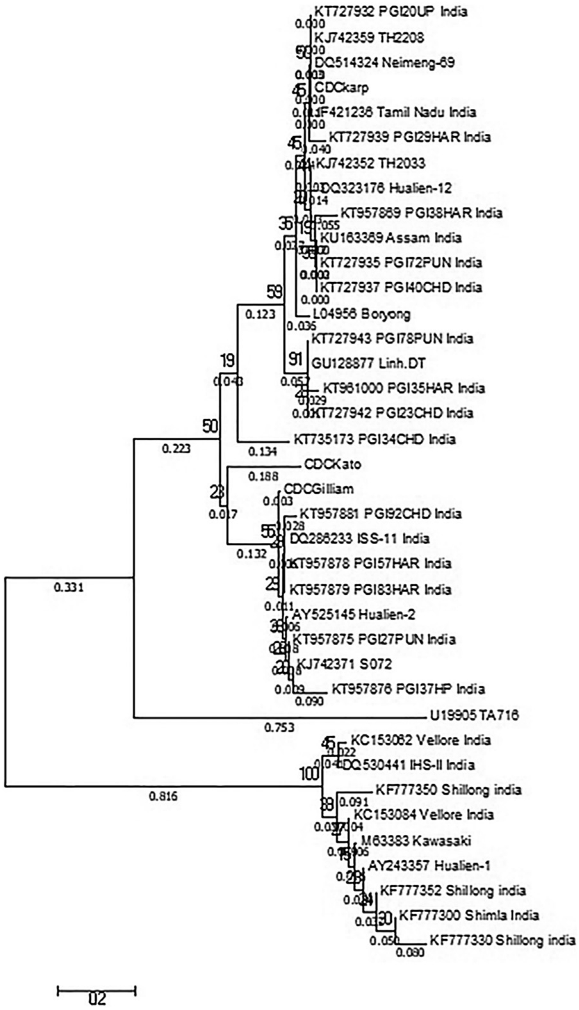

Figure 3. Evolutionary relationships of taxa of Indian strains with prototype strains by using the Neighbor-Joining method in MEGA6.

Table 1
Estimates of Evolutionary Divergence between Sequences using Maximum Composite Likelihood model by MEGA6.

		1	2	3	4	5	6	7	8	9
1	KC153084_Vellore_India	0.000								
2	DQ286233_ISS-11_India	1.251								
3	CDCGilliam	1.227	0.030							
4	CDCKato	1.245	0.248	0.257						
5	CDCkarp	1.437	0.223	0.210	0.321					
6	KU163369_Assam_India	1.393	0.188	0.176	0.322	0.030				
7	GU128877_Linh.DT	1.335	0.214	0.201	0.320	0.057	0.075			
8	L04956_Boryong	1.440	0.228	0.214	0.325	0.089	0.100	0.096		
9	KT735173_PGI34CHD_India	1.455	0.267	0.276	0.355	0.274	0.261	0.270	0.246	0.000

derived sequences, PGI34CHD closely matched with strain from Taiwan (AY222365) and others only up to 97%.

Discussion

There are more than 20 prototype strains of *Orientia tsutsugamushi* documented to date (Kelly et al., 2009). From India, there have been a few studies conducted on the genetic diversity of circulating strains (Varghese et al., 2013; Bora et al., 2018). These studies were predominantly from the north, south and extreme north-east of India. The present study documents the genetic diversity of *O. tsutsugamushi* across a very broad area of the north of the country. This present study shows that Karp-like and Gilliam-like strains were the most prevailing types. Karp-like strains also predominated in a recent study in the north east of India (Bora et al., 2018). Gilliam-types were the second most common type in our study. Jain et al in a recent study from north India also reported a predominance of Gilliam-like strains (Jain et al., 2018b). Studies from small animals and mites from central India have also found that Karp and Gilliam predominate (Sadanandane et al., 2018).

Varghese et al.'s study using PCR from eschars from south India showed a predominance of Kato-like strains, from all three regions studied. Our study could not find even a single Kato-like strain. The total number of strains studied by Varghese et al from north India was not high.8 Both the study by Varghese et al and our study sampled patients from Himachal Pradesh. It is possible that different areas of Himachal Pradesh were sampled in the two studies, resulting in different reports of diversity. Besides, our study used whole blood instead of eschars for PCR. It is postulated that some strains produce eschars less commonly than other antigenic types (Mahajan et al., 2006; Koraluru et al., 2016). Another reason for not detecting certain types by our study might be under sampling as the total number positive was 34. This might explain why we did not get Kato like strains, unlike that by Varghese et al.

We found some sequences with simlarity to Hualien strains and S072 which are related to the TA763 strains reported from Taiwan. Kawasaki type strains have been reported from south of the country by Usha et al., who also found dual infection of Karp and Kawasaki types in a few patients (Usha et al., 2016). Less commonly prevalent types have also been reported from India for example Ikeda-like by Varghese et al. (2015), Kuroki-like by Bakshi et al. (2007), JG/Saitama type by Mahajan et al. (2006) and Ikeda like by Koraluru et al. (2016). These uncommon types were not found in our study.

There are certain limitations of our study. As ours is a teriary care hospital, we generally see patients a little late in the course. The larger proportion of patients present to us in the second week of fever. The results might have been different if we had sampled more patients in the first week of fever. Also, if we had used buffy coat or eschars as samples, the PCR positivity might have been more and the genetic diversity picture might have been different. Moreover, the duration of the study period is only 6 months.

To summarize, there is considerable diversity of *O. tsutsugamushi* antigenic types in India. It is the need of the hour to conduct similar studies across the country to determine the antigenic diversity throughout the country to develop geographic-specific diagnostic tests.

Conflict of interest

None of the authors have any conflicts of interest to declare.

Ethical approval

Institute Ethic committee vide letter no. INT/IEC/2018/000023 dated 13/01/2018

Source of support

This study was funded by the Department of Medical Microbiology, Postgraduate Institute of Medical Education and Research, Chandigarh, India

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2019.04.030.

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