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Seroprevalence and Genotypic Characterization of *Orientia tsutsugamushi* in Febrile Pediatric Patients Admitted in Tertiary Care Hospital of Chennai, South India

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Abstract

Scrub typhus is one of the important vector borne illness which is largely underdiagnosed, particularly in children. It causes mild febrile illness to severe complications. More than 20 genotypic clusters are documented from various geographical regions based on sequence variations of 56kDa type specific antigen gene of *Orientia tsutsugamushi*, the causative agent of scrub typhus. Adequate knowledge about epidemiology and genetic diversity in endemic regions is an important tool for clinical management, development of diagnostic kit and vaccines. Limited studies are available based on genotypic characterization of *Orientia tsutsugamushi* in children. The present study determined the prevalence and genotypic characterization of *Orientia tsutsugamushi* in febrile pediatric patients admitted in tertiary care hospital of Chennai, South India. Both serum and blood samples were collected from 239 scrub typhus suspected febrile pediatric patient's aged between 6 months to 12 years. IgM ELISA and 56kDa nested PCR were performed on all the patient samples. Nested PCR positive samples were sequenced and analyzed for genotypic differences. Among 239 samples, 103 were positive for IgM ELISA and 35 were positive for nPCR analysis. Out of the 108 scrub typhus positive cases, 45.31% (58/128) were male and 45.05% (50/111) were female. Eschar was positive in 56.48% of patients. Pneumonia (4/108), hypotensive shock (3/108), and myocarditis (1/108) were the most common clinical complications associated with scrub typhus positive children. Karp (56.6%) was the most common genotypic cluster found in our study, followed by TA716 (33.33%), TA763 (2/30), and Gilliam (1/30).

Keywords: *Orientia tsutsugamushi*, Scrub Typhus, Eschar, 56kDa Antigen, Nested PCR

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INTRODUCTION

Scrub typhus (ST) is an emerging vector borne zoonotic disease caused by *Orientia tsutsugamushi*, an obligate intracellular gram-negative bacterium belongs to *Rickettsiaceae* family.[1-3] ST is transmitted through bite of an infected trombiculid chigger mite leading to acute febrile illness with varying clinical presentation from mild self-limiting illness to severe life-threatening complications.[3-5] The possible reason for varying clinical picture from mild to fatal disease may be due to the large variation in antigenic property and its influence towards virulence characteristics of the infecting strain.[6-8]

Orientia tsutsugamushi is responsible for one million ST cases and one billion at risk of acquiring infection annually in the endemic region. ST slowly extending from its endemic zone called "tsutsugamushi triangle" to a wider region such as Africa, South America and Arabian Peninsula.[3,7,9] In India, ST is re-emerging as an important cause of acute febrile illness with high morbidity and mortality.[5,10] The early clinical suspicion, rapid diagnosis and prompt treatment are important factors helpful in the prevention of morbidity and mortality due to scrub typhus.[11] Although the presence of eschar at the site of mite bite is pathognomic, its appearance is highly variable possibly based on the host, geographic area and type of strain.[12,13]

There are more than 20 distinct strains of *O. tsutsugamushi* based on sequence variability of the unique 56kDa immunodominant antigen, which includes Karp, Kato and Gilliam prototypes.[14,7,13] The strain distribution of *O. tsutsugamushi* varies significantly depending on geographical region.[7,15,8] Adequate knowledge about epidemiology and genetic diversity is essential for early diagnosis, development of rapid diagnostic kit and vaccines in the endemic region.[16,8,17] There have been a few studies on pediatric scrub typhus, and the available prevalence data is mostly based on serology. Hence, the present study was conducted to assess the seroprevalence and genotypes of *O. tsutsugamushi* in children.

METHODOLOGY

This is a prospective study that included pediatric patients aged 6 months to 12 years who were hospitalized for severe febrile illness at a tertiary care center in Chennai from August 2019 to February 2021. The study included patients clinically suspected for scrub typhus, who had a fever for more than three days but less than 14 days with any of the following symptoms: myalgia, nausea, vomiting, diarrhea, cough, shortness of breath, rash, eschar, lymphadenopathy, hepatomegaly, and splenomegaly. Furthermore, children having fever from causes other than scrub typhus were excluded from the study. A complete demographic, clinical and laboratory profile were collected from all the patients. The study was approved by the institution's human ethics committee, and informed consent was obtained from the children's parents/guardians prior to collecting clinical samples.

Whole blood and serum samples were collected separately for IgM ELISA and nested PCR (nPCR). ELISA was performed in serum samples, using commercially available scrub typhus IgM ELISA kit (InBios International Inc, USA) as per the manufacturer's instruction. DNA was extracted from whole blood using QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Nested PCR was used to amplify a 483bp hypervariable region of the *O.tsutsugamushi 56kDa* type specific antigen gene using previously published nucleotide primers and conditions with little modification.[18,19] The PCR condition for both the rounds were same as follows: Initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 50s, annealing at 55°C for 60s, extension at 72°C for 90s and a final extension at 72°C for 7 min. After the second round, the final PCR amplicon was resolved on 2% agarose gel and visualized using a UV transilluminator. The purified PCR amplicon was sequenced and sequences were aligned and edited using BioEdit sequence alignment editor version 7.2.5.0. Aligned sequence were analyzed for homology with *Orientia tsutsugamushi* using Basic Local Alignment Search Tool in NCBI

website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences from different countries have been obtained from the GenBank database and aligned. A phylogenetic tree was constructed using Neighbor-joining method and the evolutionary distance was calculated by Maximum composite likelihood model. The sequences of 30 isolates were submitted to NCBI GenBank with the accession numbers OL627303-OL627332.

GraphPad Prism v8.4.3 for Windows was used for statistical analysis. The Qualitative variables were presented as frequencies (percentage), whereas quantitative data such as age and days of fever were presented as Mean ± Standard Deviation (SD)/Median and Interquartile Range (IQR). The qualitative variables were compared using the Chi-square test or Fischer's Exact test, while the quantitative variables were compared using the t-test or Mann-Whitney U test, as appropriate. Multiple logistic regression was used to investigate the association between scrub typhus positive and negative patient's laboratory and clinical data. A 'P'-value less than 0.05 was considered statistically significant.

RESULTS

Clinical and laboratory findings

A total of 239 pediatric patients with acute febrile illness were screened for scrub typhus(ST) and 45.18% of children (108/239) were diagnosed with ST either by IgM ELISA or PCR. Out of 108 samples, 103 were positive for IgM ELISA, while 35 showed positive in nested PCR. Of the 108 scrub positive cases, 45.31% (58/128) were male and 45.05% (50/111) female. The average age of the patients with scrub typhus was 69.33±37.63 (in months). The youngest one diagnosed positive for scrub typhus was a 6-month-old infant. In ST positive patients, the mean duration of fever at the time of sample collection during a hospital visit was 6.99±12.29 (SD) days, with a minimum

Table 1. Clinical and Laboratory Parameters of Scrub typhus Positive patients (n=108)

Clinical and Laboratory data of Scrub typhus positive patients (n=108)			
Patient Characteristics		Laboratory Profile	
Sex		Thrombocytopenia (Platelet<100,000 cells/mm^3)	54 (50%)
Male	58/128 (45.31%)	Hyponatremia (Na<135 meq/L)	60 (55.56%)
Female	50/111 (45.04%)	Total Leukocyte count (cells/mm^3), Mean±SD	9725±4504
Age (months)	69.33±37.63	AST (IU/L), Mean±SD	104.1±168.7
Fever duration (Days)	6.991±2.29	ALT (IU/L), Mean±SD	65.73±69.23
Vomiting	57 (52.78%)		
Abdominal pain	29 (26.28%)		
Diarrhea	13 (12.04%)		
Chills/Rigors	27 (25%)		
Cough	25 (23.15%)		
Breathlessness	10 (9.26%)		
Myalgia	42 (38.89%)		
Seizures	7 (6.48%)		
Oliguria	14 (12.96%)		
Eschar	61 (56.48%)		
Rash	7 (6.48%)		
Lymphadenopathy	24 (22.22%)		
Hepatomegaly	48 (44.44%)		
Splenomegaly	36 (33.33%)		
Complications			
Pneumonia	4 (3.7%)		
Hypotensive shock	3 (2.7%)		
Myocarditis	1		

AST-aspartate aminotransferase; ALT-alanine aminotransferase; IU/L-International unit per litre; SD-standard deviation

of 4 days and a maximum of 13 days. There was no significant difference between the duration of fever between PCR positive and IgM ELISA positive patients (P=0.4204).

The clinical findings and laboratory profile of 108 ST positive patients were shown in Table 1. The most common clinical symptoms were vomiting (52.7%), followed by myalgia (38.8%), hepatomegaly (44.4%), splenomegaly (33.3%) and abdominal pain (26.85%). Eschar was observed in 56.48% of patients and commonly seen in axilla region (15/61) followed by groin, chest, face, eye, ear, neck, arm and gluteal region. The most common laboratory findings in the present study were hyponatremia (55.5%) and thrombocytopenia (50%). The most common clinical complications in ST positive cases were pneumonia (4/108) with one patient on mechanical ventilation followed by hypotensive shock (3/108) and myocarditis (1/108) (Table 2). There was no mortality in this study.

Genotypic characterization

For 35 (14.64%) samples, the target area of the *O.tsutsugamushi 56kDa* gene was amplified and sequenced. The 30 samples with good sequence reads were subjected to phylogenetic analysis and a phylogenetic tree was constructed using 30 sequences from this study and 41 reference sequences from various endemic regions (Figure). Majority of them closely associated to the Karp-like cluster (56.6%) followed by TA716–Cluster (33.33%), TA763-clusters (2/30) and Gilliam-like (1/30). The major cluster found was Karp-related and it was further differentiated into 2 clades. Clade 1 clustered with strains from Korea, China, Pondicherry, Karnataka and North India, while Clade 2 clustered with that of Vietnam, Korea, Bangladesh, Assam and Mizoram.

The Karp-related strain showed 94% to 99% nucleotide similarity with Karp reference strain AY956315.1 CDC Karp. TA716 indicated 92.8% to 93.56% similarity range with reference strain U19905.1. Two strains from the TA763-clusters were 91% identical to the reference strain U80636.1. The Gilliam strain and the reference strain DQ485289.1 were found to be 97% identical. BLAST analysis showed majority of the sequences obtained in our study were closely related to

Table 2. Multivariate analysis of variables of patients diagnosed Scrub typhus positive and Scrub typhus Negative

Variables	Odd's ratio (95% CI)	p-Value
Vomiting	1.659 (0.7703-3.636)	0.1984
Diarrhea	0.7285 (0.1853-2.505)	0.6300
Abdominal Pain	0.8067 (0.3061-2.057)	0.6565
Chills/Rigors	4.089 (1.464-11.95)	0.0081
Cough	0.5217 (0.2117-1.231)	0.1449
Myalgia	0.8904 (0.3955-1.977)	0.7762
Oliguria	3.623 (0.9801-13.71)	0.0533
Eschar	48.59 (16.78-183.5)	>0.0001
Seizures	0.7202 (0.09460-3.887)	0.7272
Breathlessness	1.142 (0.2292-5.842)	0.8706
Lymphadenopathy	3.548 (1.107-12.04)	0.0360
Splenomegaly	2.573 (1.041-6.485)	0.0416
Hepatomegaly	1.497 (0.6151-3.616)	0.3693
Total Leukocyte count	1.000 (0.9999-1.000)	0.5733
Thrombocytopenia	1.590 (0.7278-3.501)	0.2444
Hyponatremia	2.962 (1.404-6.524)	0.0053
AST	1.001 (0.9945-1.007)	0.7833
ALT	0.9974 (0.9858-1.008)	0.6438

AST- aspartate aminotransferase; ALT- alanine aminotransferase; CI-confidence interval; p-value <0.05 considered to be statistically significant

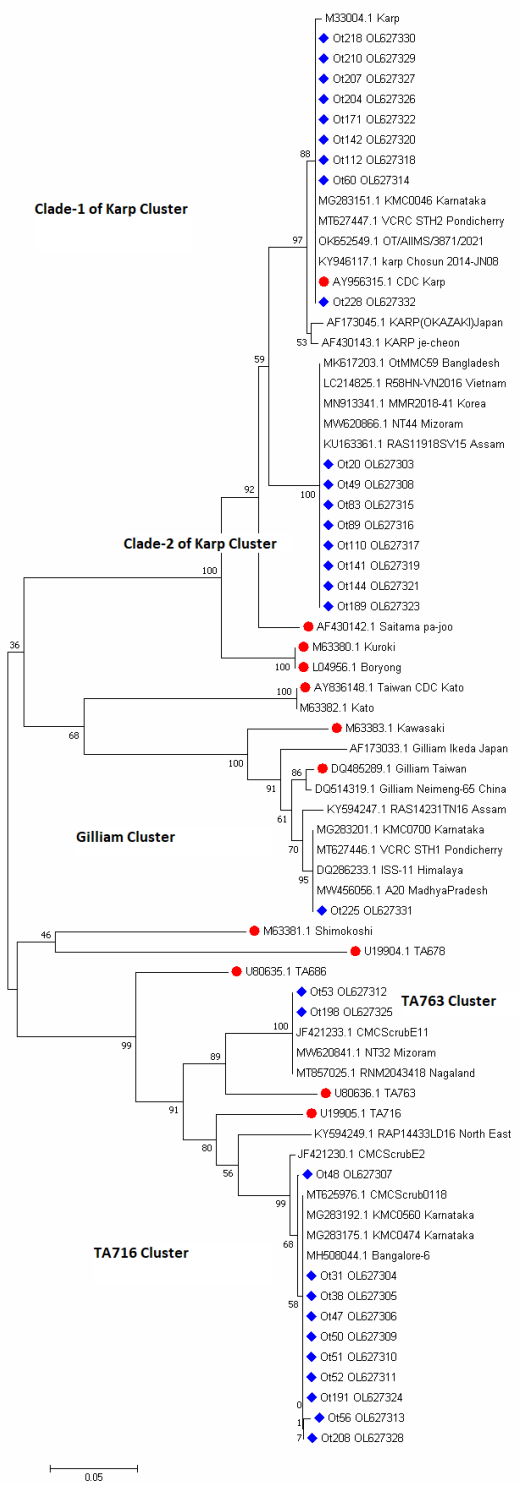

Figure. Phylogenetic analysis of partial 56kDa gene sequence of 30 strains from the present study and 41 of reference strains computed using Neighbor-Joining method, with 1000 replicate bootstrap test in MEGA 7. Diamond shaped signs indicate strains (Accession number OL627303-OL627332) of present study and circle signs indicate prototype strains of *O. tsutsugamushi*

Table 3. Clinical and laboratory parameter of two major genotype Karp and TA716 from this study

Clinical Data	Karp (n=17)	TA716 (n=10)	p value
Days of fever	7.118±2.690	6.600±2.319	0.6034
Vomiting	11(64.7%)	7(70%)	0.999
Abdominal Pain	5(29.41%)	2(20%)	0.6784
Chills/Rigors	5(29.41%)	1(10%)	0.3625
Cough	2(11.76%)	1(10%)	0.999
Myalgia	7(41.18%)	2(20%)	0.4059
Diarrhoea	1(5.88%)	0	0.999
Oliguria	2(11.76%)	0	0.5157
Breathlessness	1(5.88%)	1(10%)	0.999
Seizures	1(5.88%)	0	0.999
Eschar	10(58.82%)	4(40%)	0.4401
Rash	1(5.88%)	0	0.999
Lymphadenopathy	3(17.65%)	4(40%)	0.3648
Hepatomegaly	9(52.94%)	1(10%)	0.0415
Splenomegaly	5(29.41%)	4(40%)	0.6831
Laboratory profile			
Thrombocytopenia (Platelet<100,000/mm^3)	6(35.29%)	7(70%)	0.1201
SGOT/AST(IU/L)	91.12±79.89	106.5±137.1	0.7512
SGPT/ALT(IU/L)	69.53±43.77	74.40±71.86	0.849
Hyponatremia (Na<135meq/L)	11(64.71%)	3(30%)	0.1201
WBC(cells/mm^3)	10141±5346	8780±3208	0.4162

strains from Vellore, Pondicherry, Karnataka, Mizoram, New Delhi and Bangladesh.

Clinical characteristics of the scrub typhus genotypes

The difference in the clinical and laboratory parameters were analyzed between the two major ST genotypes (Karp and TA716) observed in this study (Table 3). We found significant difference in hepatomegaly between Karp and TA716 ('p' value 0.0415) with higher prevalence of hepatomegaly in Karp compared to TA716.

DISCUSSION

Scrub typhus is a rickettsial infection that is re-emerging in the Indian subcontinent; however, it is an under-recognized cause of febrile illness in children.[20,21] The current study investigated the prevalence and genotypic characteristics associated with ST in pediatric patients. In India, there has been limited study on the prevalence and genetic characteristics of scrub typhus in pediatric patients. The prevalence rate of ST in our study was 45%, which is comparable to the study conducted in Odisha, where 48.7% of ST was reported in the pediatric population.[22] In the present study, we observed the Karp-like was the most common genotype, followed by TA716 related, TA763 related and Gilliam. The Karp-related strains were the predominant genotype observed in Southeast Asia (Thailand, Vietnam, Cambodia and Taiwan), East Asia (Japan, China), Northeast India and Bangladesh.[7,23-25] A study from eastern region of India reported the occurrence of Kato as the single most prevalent strain.[19] In a study from North India, Gilliam has been identified to be the most common genotype, followed by Karp-like strains.[26] Kato-like strains have been observed to be widespread throughout the country.[27] Studies from South India revealed a variable pattern of *O. tsutsugamushi* genotypes; one study identified Karp as the main strain[28] while another identified Gilliam as the dominant strain.[29] Kato was found to be a prevalent strain in a study from South India.[8] The genotypes such as Kato, Ikeda, Kawasaki, Boryoung which were reported from various regions of South India[8,30,31,29] were not found in our study. There has been an increased evidence of TA763 genotypes across India. The TA763 genotype originally found in Thailand and present predominantly across the Southeast Asia.[7] The TA763 genotypes observed in the present study was found closer to strains isolated from Vellore, Bangalore, Mizoram and Haryana. The diversity in the distribution of genotype in various geographical region might be possibly due to the difference in vector mite species present in the particular area. The genus of *Leptotrombidium* were found to be the major vector in transmitting scrub typhus throughout the endemic region,[7] but studies from different geographical regions observed variation in the dominant mite species *O. tsutsugamushi* genotype. In a Korean study, Karp was observed within regions where *L. pallidum* was prevalent, while Boryoung was identified in regions where *L. pallidum* and *L. sutellare* were prevalent.[6] A Japanese study observed a potential role for *L. pallidum i*n the transmission of Karp and *L. palpale* in Shimokoshi genotypes.[32] In India, *L. deliense* was found to be dominant species in Tamil Nadu and Andhra

Pradesh, and Pondicherry,[33,34] *Ornithonyssus bacoti* as a common vector species in Central India[35] and *Schoengastiella ligula* was isolated in rodents from Darjeeling[36] and Vellore.[33] In a study from Central India reported the Karp as the predominant strain and the *L. deliense* as the vector species involved in ST transmission.[37] A study from North Tamil Nadu identified *O. tsutsugamushi* in multiple mite species with *Aschoschoengastia* spp. as the predominant one and also found TA716 and Kato genotype from pooled mite sample.[38]

The present study, which was conducted in one of Chennai's tertiary care hospital, revealed a higher prevalence rate of ST in this urban area surrounded by semi-urban and rural belts. In a study from Eastern India reported highest percentage of scrub positive cases from urban area (75.4%) compared to 24.5% at rural.[39] Another study at Nagpur, Central India, reported 52.14 % prevalence of ST from urban region compared to 47.85% of rural region.[40] The possible reason for increase in ST prevalence in urban area may be due to globalization, climate change, larger influx of population into urban region, and deforestation of adjacent rural region for city expansion led to displacement of vectors as well as rodents from one place to other[41]; that may widen the endemic area further.[19]

Scrub typhus is largely under-diagnosed in India as the clinical symptoms of ST are nonspecific and mostly indistinguishable from other tropical febrile illness like malaria, dengue fever, typhoid fever and leptospirosis. The presence of eschar is an important clue for the initial diagnosis of ST.[42,43] However, their presence varies widely in the endemic region, ranging from 7-97%,[44] and this difference may be influenced by the factors like skin color, physical examination done by the investigator and strain variation.[42,12,8] The eschar prevalence was found to be 23.57%, 6.1%, 14%, 60% from central, eastern, north and south regions of India.[40,39,17,45] In Vietnam the prevalence of eschar was found to be 65%.[46] A study conducted at southwestern region of Korea reported a highest proportion in ST patients confirmed with IFA.[12] Compared with Karp cluster, eschar was observed in 97% of patients infected with Boryoung strain.[13] The pathognomic eschar found in this study was 56.4%, which was closer to a previous study from South India.[47] Eschar was found to be a substantial predictor of scrub typhus in our study, with a significant p-value of 0.0001. In this study, eschar was detected in patients infected with the Karp and TA716 clusters but was completely absent in patients infected with the TA763 and Gilliam genotypes. Splenomegaly is one of the important clinical characteristics for ST which distinguishes it from dengue.[48,49] Our study reported 33.33% of splenomegaly in ST patients with significant 'P'-value. The other important predicting factors of ST with significant 'P'-value<0.05 found in this study were chills/ rigors, lymphadenopathy and hyponatremia using multivariate analysis. In our study, the clinical complications of ST included pneumonia, hypotensive shock and myocarditis. However, meningoencephalitis and acute kidney injury were found to be the important complications of ST in our region.[50-52] Delay in diagnosis and treatment leads to Multiorgan dysfunction syndrome and increase in the mortality rate.[40,5] A fatality rate of 6.6% was observed in a previous study and the author cited delayed presentation to hospital and delayed beginning of effective therapy as possible reasons for mortality in ST cases.[26] In a study from South India the author reported a decreased trend in mortality rate in Scrub typhus cases from 14.6% to 7.6% during a four-year period,[5] as well as an overall reduction of 14% to 9% when compared to his previous study.[43] In our study we observed zero mortality in ST positive patients. Increased awareness among physicians and point of care diagnosis and implementation of empirical treatment might be the reason for lesser complications and zero mortality observed in this study. However, the genetic diversity in different geographical regions and its role in virulence could not be ruled out. Several studies on human and animal models examined the effect of *O. tsutsugamushi* genotypes on clinical symptoms and the degree of sequelae. In a study conducted using mice model the difference in virulence property was observed in the genotypes of *O. tsutsugamushi.*[53] Clinical pictures like general weakness and conjunctival injection along with the laboratory parameters ESR and plasma fibrinogen were significantly higher in patients infected with Boryoung strain when compared to Karp clusters.[13] Whereas, low level of platelet count and albumin, higher rates of edema and increased level of AST

were observed in patients infected by Karp cluster compared to that of Kato and Gilliam genotypes.[46] We compared the clinical variations among Karp and TA716, the two major genotypes observed in this study and found that hepatomegaly was significantly higher in Karp infected cases than in TA716 infected cases. However, no significant differences in liver enzyme levels (AST and ALT) were found in patients. Decreased platelet level was found to be prominent in TA716 (70%) compared to Karp (35.29%), similarly hyponatremia was higher in Karp strain (64.71%) than TA716 (30%),but they were statistically not significant. In present study, the complications like myocarditis (one patient) and hypotensive shock (1/3) were observed in patient infected with Karp cluster and not in TA716, TA763 and Gilliam genotypes.

In the present study, *O. tsutsugamushi* DNA was found in 35/239 (14.64%) whole blood samples which was in contrast to the previous studies.[17,24,27] Many factors can possibly influence the sensitivity of 56kDa nested PCR while using whole blood, such as the presence of PCR inhibitors in the blood, reduced bacterial load due to antibiotic therapy and/or immune response against *O. tsutsugamushi* and intermittent bacteremia.[54,55,44] The sensitivity of 56kDa nPCR can be increased by using eschar and buffy coat sample instead of whole blood. The sensitivity has been reported to be 86.5% (eschar) and 83.2% (buffy coat) with 100% specificity.[55,56] The inclusion of eschar samples might have increased the chance of PCR positivity in this study. However, variation in eschar distribution across geographical regions, as well as the influence of genotypes on eschar frequency, must be considered. Buffy coat is also an excellent sample for increasing the sensitivity of *56*kDa nPCR. However, as the quantity of blood taken from pediatric patients was insufficient to separate buffy coat, *56*kDa nPCR based on buffy coat could not be performed in this study.

CONCLUSION

The various studies evidenced increase in the prevalence of scrub typhus across the country and it was largely under-recognized in pediatric patient. The present study showed the prevalence of Scrub typhus in pediatric patients and genotypic distribution of *Orientia tsutsugamushi* in Chennai, South India. The study on prevalence of scrub typhus and genetic diversity in the endemic region is not only useful in epidemiological purpose but also for the improvement of diagnostic accuracy, vaccine development and understanding virulence and its role on variation in the clinical picture. Increased awareness, point-of-care diagnosis, and evidence-based empirical treatment may have contributed to lower mortality and complications in our study. The role of genotype diversity in virulence cannot be ruled out. We found no evidence of strains such as Kato, Kawasaki, or Boryoung, which have been described in prior studies of the South Indian region. As eschar and/or buffy coat were utilized for 56kDa nested PCR instead of whole blood in this investigation, the final result may have changed. We have observed the clinical difference between genotypes at a smaller level only due to less number of PCR positive cases. In the future, a multicenter investigation with a large number of samples could provide the accurate spectrum of prevalent genotypes in Tamil Nadu, South India, and their significance in virulence.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

PD, RM, SK and PS designed the study. RM, SK and PS performed the experiments. PD supervised the experiments. PD, RM, SK and PS analyzed the data. RM, SK and PS wrote the manuscript. PD reviewed the manuscript. All authors read and approved the final manuscript for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This study was approved by the Institutional Humans Ethics Committee, Dr. ALM PG IBMS, University of Madras, Chennai, India, with approval number UM/IHEC/F.RM/2021-XI.

INFORMED CONSENT

Written informed consent was obtained from the participants before enrolling in the study.

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