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ORIGINAL ARTICLE

Molecular-based study of scrub typhus in Kerala, South India from 2014 to 2021: a laboratory-based study

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Abstract

Scrub typhus (ST) is a neglected acute, febrile, infectious disease caused by the intracellular parasite *Orientia tsutsugamushi*, a gram-negative coccobacillus of the family Rickettsiaceae. Early and precise diagnosis is crucial to reduce the risk of developing disease complications. However, IgM antibody enzyme-linked immunosorbent assay (IgM ELISA) and indirect immunofluorescence assay (IFA) remain essential for diagnosis. However, it could be more helpful for early diagnosis due to the need for uniformity of approach in the diagnostic accuracy studies to determine appropriate ELISA cut-offs for various geographic locations. Hence, we aim to study the *O. tsutsugamushi* type-specific 56 kilodalton (kDa) protein gene using nested PCR (nPCR) and DNA sequence analysis as a molecular marker for early diagnosis. Out of 10,439 suspected cases, 1147/10,439 (11%) patients were positive for IgM ELISA. A total of 1044/10,439 (10%) samples were randomly tested after nPCR and compared with IgM ELISA results and DNA sequence analysis. Using nested PCR and IgM ELISA methods, 13% (134/1044) and 12% (125/1044) of the samples were positive, respectively. The serology method could not replicate the substantial number of positive cases demonstrated by nPCR; therefore, significant mutual exclusivity of the two techniques requires further investigation. Furthermore, our phylogenetic analysis revealed a clustering of isolates with Karp-related strains, providing insight into the transmission dynamics. Therefore, molecular diagnostic methods may aid in the early diagnosis of infection and enable prompt treatment of ST in endemic regions. Our results show that IgM ELISA can provide complete diagnostic advantages in conjunction with nPCR and can be an essential tool for accurate diagnosis. In addition, the DNA sequencing analysis of the samples showed that Karp-related strains were the main strains. Furthermore, research with samples from various regions in combination with the entire genome sequencing of *O. tsutsugamushi* is required to understand the infection mechanism better and develop robust early detection methods.

Keywords Scrub typhus · *Orientia tsutsugamushi* · IgM antibody enzyme-linked immunosorbent assay · Nested polymerase chain reaction · Sanger sequencing

Introduction

Scrub typhus (ST) is an acute, febrile, infectious illness caused by the obligate intracellular gram-negative bacterium *Orientia tsutsugamushi* (formerly known as Rickettsia tsutsugamushi). The World Health Organization (WHO) listed it as one of the Asia–Pacific region's most under-recognized, neglected, and severe public health problems. The disease has become a significant health problem in India (WHO 1999; Xu et al. 2017; Saraswati et al. 2018; Bonell et al. 2017: Paris et al. 2013). The contagious zoonotic disease is spread by mites known as chiggers. Chigger is the only form in the life cycle of the mite of the genus *Leptotrombidium* that bites warm-blooded animals, whereas the other life forms, i.e., nymphs and adults, live on soil and plants. Transfer of the causative agent, *O tsutsugamushi*, at different stages of the agent's vector transmission from adult to offspring (Walker et al. 1975). A re-emerging infectious disease worldwide and is prevalent in many parts of India (Ming et al. 2020; Prakash 2017). Early and precise diagnosis is crucial to

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reduce the risk of complications and prevent mortality and initial treatments (Basu and Chakravarty 2022). The WHO recommended prophylactic treatment under exceptional circumstances in endemic areas (Olson et al. 1980). Clinical diagnosis is unreliable for identifying ST, unless a tick eschar is present, which is almost pathognomonic for the disease in Southeast Asia. ST symptoms are difficult to delineate from other febrile illnesses due to similar clinical manifestations. It is often misdiagnosed due to a lack of accurate and user-friendly diagnostic tools (WHO 1999). As a result, laboratory confirmation is not widely available, and the diagnosis is frequently missed in clinical practice. A combination of culture-paired serology and polymerase chain reaction has been proposed as the gold-standard method for detection. The antigenic diversity of the three prototype strains is Gilliam, Karp, and Kawasaki (Shishido, 1962). Later, additional antigenic types were reported, including Kawasaki, Kuroki, and Shimokoshi. Other distinct serotypes are present in the tsutsugamushi triangle, and the reason for outbreaks may be due to antigenic heterogeneity (Yamamoto et al. 1986; Ohashi et al. 1990; Tamura et al. 1984). Traditionally, ST diagnosis relied mainly on serologic tests like indirect immunofluorescence assay (IFA). The disease could be retrospectively diagnosed in seroconversion cases or a > fourfold rise in antibody titers between acute-phase and convalescent-phase serum specimens. Although serology has remained a base for diagnosis, it does not help with first-line treatment (Phetsouvanh et al. 2013). Serologic tests are not helpful for acute early diagnosis because they usually become positive only during recovery. A PCR assay was developed to detect the *O. tsutsugamushi* Sta56 gene in blood samples or isolates from patients (Furuya et al. 1993; Kelly et al. 2009). However, the test often gave false-negative results because hemoglobin and other blood components may inhibit PCR amplification. Real-time PCR assays are sensitive as standard PCR, but they are more rapid and can give quantitative results. Nested PCR (nPCR) offers an advantage in early and viable alternatives (Singhsilarak et al. 2005; Kala et al. 2020).

The present study aimed to improve ST's epidemiology and characteristics in Kerala using multiple diagnostic assays. To investigate diagnostic assay appropriately for southern parts of India, we also analyzed the Karp-related strain's genetic factors to detect ST of 56 kilodalton (kDa) gene using nPCR, parallel evaluating serology with IgM enzyme-linked immunosorbent assay (IgM ELISA) method and their clinical utility in early diagnosis. The present report is the first systematic study to determine the real impact of ST diseases in Kerala, India.

Materials and methods

In the present study, clinical samples are from the patients (male or female ≥15 years of age) who visited Thiruvananthapuram Medical College from January 2014 to September 2021, and the Institutional Ethical Committee of RGCB approved the study (No. IEC/LMMD/17/21). Inclusion criteria were those within 4 weeks of fever (with an eschar) onset with febrile illness suspected of having an early stage of ST infection defined as per WHO criteria (acute onset of fever and change in mental status or new onset of seizures). Exclusion criteria were cases with fever, for which the cause was already known and had no eschar, pregnancy or breast-feeding, and patients with an established infection (diagnostic test required), e.g., acute malaria, dengue, leptospirosis, typhoid, Japanese encephalitis, and simple febrile (seizures) (WHO 2006). Information, including demographics, medical history, and clinical and laboratory findings, were collected into a case record form. The IgM ELISA processed and blindly tested the EDTA blood/serum samples of 10,452 cases. Out of 10,439 samples, 10% randomly chose some negative, positive samples ($n = 1044$) for nPCR assay validation.

IgM ELISA

The serum was separated by centrifugation at 2500 g for 10 min, and clots were stored at −70 °C. All serum samples from the patients were tested by IgM antibody to *O. tsutsugamushi* and were detected using ST Detect™ Kit, InBios International, USA, containing the recombinant p56kDa type-specific antigens. *O. tsutsugamushi* Karp, Kato, Gilliam, and TA 716 strains according to the manufacturer's instruction. Optical density (OD) was measured by HumaReader HS, ELISA reader, and OD > 0.50 was considered positive. The cut-off was calculated following recommendations for determining the endemic cut-off titer in the kit protocol. The cut-off calculated from the healthy volunteer was mean OD (0.23) + 3 standard deviations (0.09) = 0.50.

Detection of the 56-kDa type-specific antigen (TSA) gene by nPCR

Nested PCR was performed to detect *O. tsutsugamushi* DNA in the DNA extract, as described previously (Furuya et al. 1993; Horinouchi et al. 1996; Kim et al. 2011). DNA was extracted from the blood sample (buffy coat) by using QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany). For each patient, 200 µl of the sample was used for DNA extraction. Extracted DNA from each specimen was eluted into 100 µl of Tris EDTA (TE) buffer for

molecular analysis. After extraction, sample DNA was stored at −80 °C. The nPCR primers were designed from consensus genome regions of Karp, Kato, Gilliam, and Boryong strains obtained from GenBank (http://www.ncbi.nlm.nih.gov/) and purchased from Sigma–Aldrich (Bangalore, India). The first amplification mixture containing 2.5 µl of extracted DNA was used as a template in the first amplification step. One-tenth of the total volume from the first amplification was used for the second amplification. A total of 25 µl of the PCR reaction mixture contained template DNA, 400 nM of each primer, 200 µM of dNTP, 1× PCR buffer, and 0.5 units of PrimeSTAR® Max DNA Polymerase (TaKaRa India). PCR reactions were performed using the following reaction conditions in Veriti™ 96-Well Fast Thermal Cycler (Thermo Fisher Scientific Inc.). The first round of nPCR for the 56-kDa gene was performed under the following conditions that involved an initial denaturation at 94 °C for 5 min, 30 cycles for 30 s at 94 °C, 30 s at 63 °C, and 1 min at 72 °C, with a final extension for 7 min at 72 °C. Five pmol/µl of each primer (STf and STr) was used (as shown in Supplementary Table 1). The second round of nPCR for the 56-kDa gene was performed using the first round PCR product as the template DNA and 5 pmol/µl of each primer (STNf and STNr), and the conditions are like the first round. Amplified products were electrophoresed in 2% agarose gels and stained with ethidium bromide solution for visualization using the E Gel imager (Thermo Fisher Scientific Inc.) gel documentation system (Fig. 1).

Sanger sequencing and phylogenetic analyses of the 56-kDa TSA gene

We designed in-house primers targeting a 400 bp fragment of the *O. tsutsugamushi* 56-kDa TSA gene, including the variable regions. Samples positive for *O. tsutsugamushi* were subjected to PCR amplification before sequencing. Amplification products were separated by electrophoresis on a 1.5% agarose-tris–borate-EDTA gel. PCR products were Sanger sequenced using the Big Dye Terminator kit (Applied BioSystems, USA) on an ABI PRISM 310 DNA Sequencer (Applied Biosystems, Hitachi, Japan). The sequences were analyzed using the ABI PRISM DNA Sequencing Analysis software version 3.0 (Applied BioSystems) and compared to sequences available in GenBank using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov).

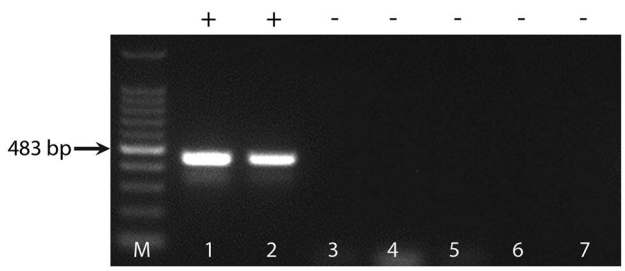

Fig. 1 Representative agarose gel electrophoresis of amplified DNA by nested polymerase chain reaction targeting 56-kDa protein type-specific antigen of *O. tsutsugamushi*. Lane M, 100 bp DNA ladder; lane 1, positive control; 7, negative control; 2–6, clinical samples. Sizes of a DNA length marker (M) are shown in base pairs

A neighbor-joining tree of 56-kDa type-specific antigen genes was constructed with the Kimura two-parameter model using sequenced samples of the current study, and reference strains are Karp, Boryong, Kato, Gilliam, and Kawasaki. To estimate the reliability of the phylogenetic trees, 1000 bootstrap replicates were performed (Tamura et al. 2001; Kelly et al. 2009).

We used MEGA 4.0 software for the phylogeny reconstruction (Kumar et al. 2008). Homologies among the genotypes were calculated using the Kimura two-parameter model (Lim et al. 2015a). Phylogenetic trees were assessed using the neighbor-joining method cluster analysis with 1000 bootstrap replications (Fig. 2).

Statistical analysis

All statistical analyses were performed with SPSS version 17.0 (SPSS Inc., Chicago, IL). The risk factors for ST were identified using univariate and multivariate conditional logistic regression models. Statistical tests were two-sided, with significance defined as *p*-values less than 0.05. For all study variables, descriptive statistics were obtained, and categorical data expressed as percentages and continuous data were summarized using mean and standard deviation. The sensitivities, specificities, PPV, and NPV of serological and molecular assays were calculated using MedCalc for Windows, version 13.3 (MedCalc Software, Ostend, Belgium). Kappa statistics were calculated to determine the degree of agreement between the assays.

Results

A total of 10,452 samples were obtained from patients with suspected ST from the Thiruvananthapuram Medical College, Kerala, and Southern India. Of these 10,452 specimens, 13 (10,439) were excluded due to exclusion criteria about age and quality of DNA. The patients from whom these samples were collected and the demographic and clinical manifestations were compared with nPCR and IgM ELISA results. A significant number of patients with nPCR and IgM ELISA presented with age, sex, and symptoms are tabulated in Table 1.

IgM ELISA

Among 10,439 samples, the age group of ≥ 20 years and ≤ 20 years showed 9.7%, and 1.5% respectively. Males

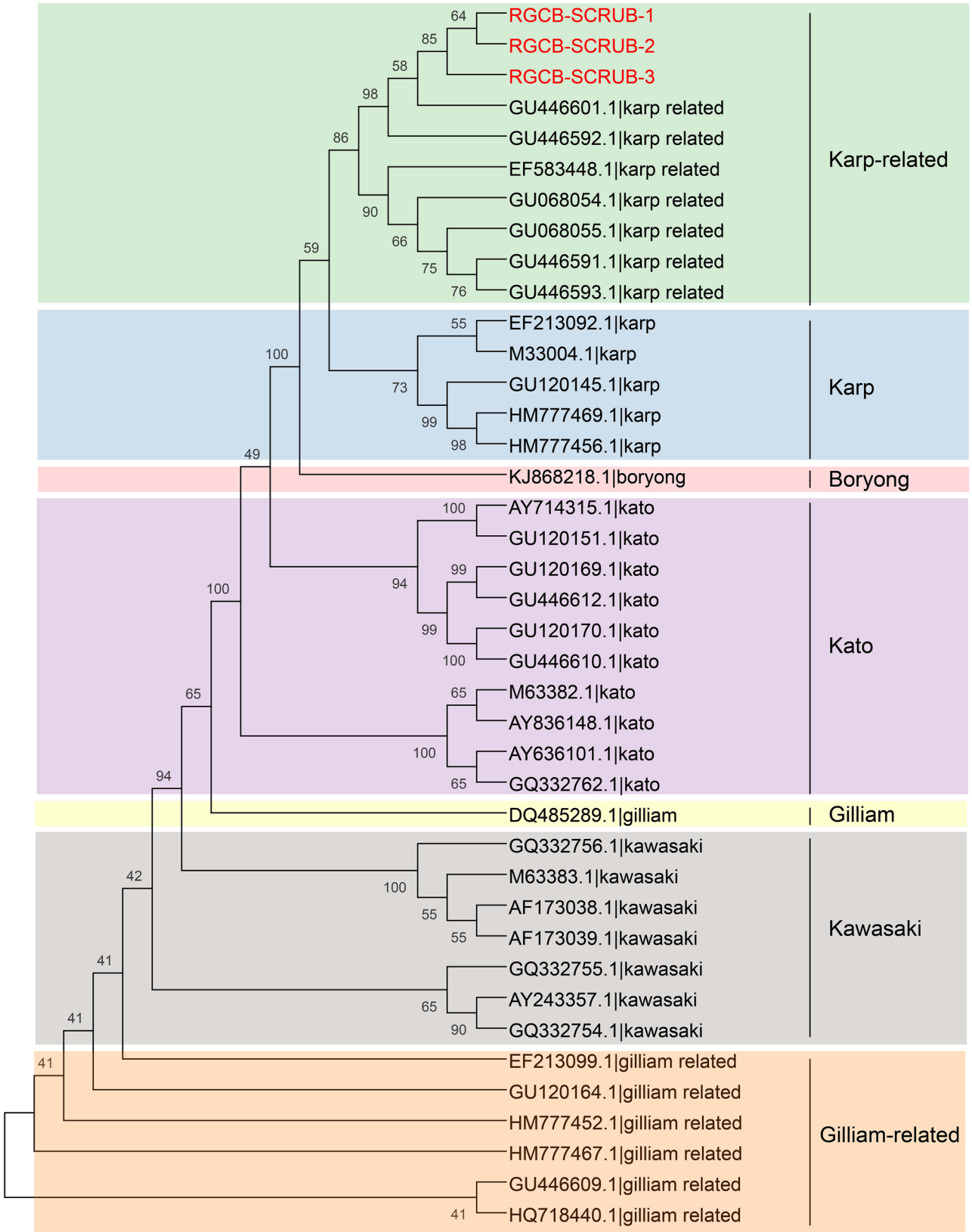

Fig. 2 Neighbor-joining tree of *Orientia tsutsugamushi* strains constructed based on sequence homologies of 56-kDa type-specific antigen genes of the current study samples with the reference strains. The numbers at nodes indicate bootstrap values. The branch labels highlighted in red are the samples used in this study

Table 1 Results of scrub typhus nPCR and IgM ELISA compared with clinical manifestations of patients (2014–2021)*

Characteristics	nPCR				IgM ELISA			
	nPCR positive ($n=134$)	nPCR negative ($n=910$)	Total ($n=1044$) N (%)	p**	IgM positive ($n=1147$)	IgM Negative ($n=9292$)	Total ($n=10,439$) n (%)	p**
Age (years)								
≤20 years	13	188	201 (19.25)	0.05235	156	2100	2256 (21.6)	2.2e-16
≥20 years	121	722	843 (80.74)	0.01707	1020	7163	8183 (78.4)	2.2e-16
Sex								
Male	118	645	763 (73.0)	0.0002737	707	6314	7021 (67.3)	2.2e-16
Female	16	265	281 (27.0)	0.001315	440	2978	3418 (32.7)	2.2e-16
Symptoms								
Fever								
≤5 days (3–5)	121	412	533 (51)	0.0001887	941	6421	7362 (70.5)	2.2e-16
≥5 days (6–12)	20	215	325 (31)	0.7901	206	2871	3077 (29.5)	2.2e-16
Headache	112	197	309 (30)	3.947e-05	973	5412	6385 (61.1)	2.2e-16
Cough	124	168	292 (28)	1.816e-07	1011	5971	6982 (66.8)	2.2e-16
Body aches and muscle pain	117	141	251 (24.02)	8.204e-05	874	2501	3375 (32.3)	2.2e-16
Eschar	116	19	135 (13)	4.119e-11	451	2984	3435 (32.9)	2.2e-16
Vomiting	94	45	139 (13.31)	0.06447	521	2245	2766 (26.4)	2.2e-16
Enlarged lymph nodes	51	12	63 (6.03)	5.617e-08	325	124	449 (4.3)	2.2e-16
Rash	61	12	73 (7.0)	0.0006871	312	142	454 (4.3)	1.481e-15

nPCR nested polymerase chain reaction, *IgM ELISA* antibody enzyme-linked immunosorbent assay

*Includes 1044 patient samples for nPCR and 10,439 patients samples for IgM ELISA

**p significance was defined as less than 0.05 (test of association was performed using Pearson's chi-square test)

showed ST positivity at 6.8%, whereas females were at 4.2% respectively. Patients with fever ≤ 5 (3–5 days) show 9.0%, which is four-fold higher than ≥ 5 (6–12 days) as shown in Table 1, and an IgM-positive finding was detected in 125 patients (12%) (Table 2).

nPCR

Among 1044 samples, results revealed that in the age group of ≥ 20 years, 11.6% showed the highest risk of ST. Males showed ST positivity of 11.3%, whereas females were 1.5%.

Table 2 Performance of IgM ELISA and nPCR individual assays in ST diagnosis and measure of agreement*

Test	True positive	True negative	Sensitivity (95% CI[4])	Specificity (95% CI)	PPV[1] (95% CI)	NPV[2] (95% CI)	*k* value[3]
IgM ELISA							
Predicted positive	110	20	82%	97%	84%	97%	0.80
Predicted negative	24	890					
Total	1044						
nPCR							
Predicted positive	100	0	74%	100%	100%	96%	0.83
Predicted negative	34	910					
Total	1044						

PPV positive predictive value, *NPV* negative predictive value, *kappa value* calculated to determine the degree of agreement between the assays, *CI* confidence interval

*The sensitivities, specificities, PPV, and NPV of serological and molecular assays were calculated using MedCalc for Windows, version 13.3

Patients with fever≤ 5 (3–5 days) show 11.6%, which is fivefold more significant than ≥ 5 (6–12 days), as shown in Table 1, and nPCR results showed 13% (134/1044) positivity (Table 2). The primers for the nPCR were designed from consensus genome regions of the Karp, Kato, Gilliam, and Boryong strains. The detection of the 56-kDa PCR assay primer specificity was checked using various other bacterial and viral DNAs. The primers were tested using genomic DNA (1:1, 1:10, 1:100) from 14 positive ST and four non-ST clinical samples. nPCR of the type strains did not give rise to bands other than *O. tsutsugamushi* (Table 3). The nPCR assay detected all ST-positive samples whereas non-ST were negative (Table 3). The amplification product of the target DNA in 2% agarose gel electrophoresis showed the 483 bp DNA fragment from the patients in lanes 2 and 3–7. Still, no DNA amplification was observed in the clinical samples and negative control (Fig. 1).

nPCR and IgM ELISA

To determine the combination of diagnostics that would give the highest sensitivity for detecting the infection, we performed a descriptive type of statistical analysis and checked the number of samples testing positive by IgM ELISA, and nPCR (56 kDa). Diagnostic capabilities of individual tests were measured by determining the diagnostic parameters such as sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) and measuring the agreement (kappa coefficient) as shown in Table 2. Among the individual tests, nPCR and IgM showed a specificity of 100% and 97%, which were quite close to that of IgM ELISA (97%) possibly due to the higher cut-off followed in the present study. IgM ELISA exhibited a sensitivity of 82%, with a measure of agreement (*k* value) of 0.8, whereas nPCR sensitivity was 74%, with a good measure of agreement of 0.83.

Table 3 Primer specificity testing using ST-positive non-ST clinical samples

Sl. no	Name of the sample	Results of nPCR*		
		1:1	1:10	1:100
ST-positive clinical samples**				
1	Sample 1	+	+	+
2	Sample 2	+	+	+
3	Sample 3	+	+	+
4	Sample 4	+	+	+
5	Sample 5	+	+	+
6	Sample 6	+	+	+
7	Sample 7	+	+	+
8	Sample 8	+	+	+
9	Sample 9	+	+	+
10	Sample 10	+	+	+
11	Sample 11	+	+	+
12	Sample 12	+	+	+
13	Sample 13	+	+	+
14	Sample 14	+	+	+
Non-ST clinical samples***				
1	Sample 1	−	−	−
2	Sample 2	−	−	−
3	Sample 3	−	−	−
4	Sample 4	−	−	−

*Genomic DNA serial dilutions (1:1, 1:10, and 1:100)

**ST-positive, PCR positive of ST

***Non-ST, dengue, typhoid, and malaria positive samples; (−), not detected; (+), detected

Sequencing and phylogenetic analysis

We confirm the PCR-amplified product using BLAST as a 56-kDa protein type-specific antigen. Our isolates clustered with at least 98% identity with the representative strains. Furthermore, by reconstructing the phylogenetic tree of the 56-kDa protein type-specific antigen gene, we found that our isolates cluster with the Karp-related strains that are responsible for causing 50% of infections. Similarly, other studies in India reported Karp and Kato strains, indicating the transmission of strains across different geographic locations.

Discussion

Scrub typhus is a severe public health disease burden in the Asia–Pacific region. It has been under-reported in India and neighboring countries, such as Pakistan, Myanmar, and Nepal. However, more attention is needed to monitor and report cases, recordings, vector control activities, and deaths. The diagnosis is challenging because the manifestations of the disease are similar to those of other febrile diseases. Delays in diagnosis can lead to severe complications such as acute respiratory distress syndrome, septic shock, and multisystem organ failure, often causing death in patients. Therefore, timely and accurate laboratory tests must confirm an effective diagnosis. In the present study, nPCR and IgM ELISA tests were used to accurately detect ST infection in the acute phase of the disease.

The IgM ELISA was based on the combination of recombinant antigens of Karp, Kato, Gilliam, and TA 763 genotypes and was observed to have a sensitivity of 82% and a specificity of 97% (Table 2). The usefulness of IgM ELISA in ST diagnosis was described (Dasch et al. 1979). Similar sensitivity and specificity were observed in another study (Prakash et al. 2006). However, a study from Thailand observed better sensitivity but slightly lesser specificity of 93.0% and 94.0% respectively at a cut-off titer of 1:400

(Coleman et al., 2002). However, careful consideration must be given to determine the cut-offs OD before the utility of IgM ELISA.

However, other studies reported similar findings. Of the 370 acute encephalitis syndrome (AES) patients tested, 63% had IgM positive for ST. Another study reported that of 407 AES patients, 65% had positive ST IgM (Murhekar et al. 2016). The primary constraint of this study was that antibiotic-treated patients would bring down the sensitivity of PCR. This study recommends that IgM ELISA can diagnose infection after 5 days of fever onset, while nPCR is used for diagnosis within 5 days of fever and confirmation in untreated cases. Hence, in conjunction with nPCR, IgM ELISA can provide a complete diagnostic advantage and be employed as a diagnostic tool to diagnose ST in endemic areas.

This study demonstrated that the diagnostic performance was better for detecting ST. nPCR showed a sensitivity and specificity of 74% and 100%, respectively. These results are in correlation with the results of Korean researchers, where a sensitivity of 82.2% and specificity of 100% (Kim et al. 2006) and other studies also show similar findings (Patricia et al. 2017; Lim et al. 2015a, b; Paris et al. 2011). The sensitivity of PCR for detection at the early stage of infection is 76% (10 positive cases out of 13), and for ELISA, it is 23% (8 out of 13) (Rawat et al. 2018; Gupta et al. 2016; Murai et al. 1992). Additionally, six nPCR cases were negative by positive for IgM ELISA. This is surprising, and possible reasons could be the differences in the genetic nature of the infecting isolate.

Scrub typhus may fail to be diagnosed based on serum antibody titers during the acute and convalescent stages because antibody formation and a fourfold or more significant increase in specific antibody titer require several weeks to occur (Kim et al. 2017). In contrast, PCR can detect *O. tsutsugamushi* DNA in blood from the onset of symptoms, thus allowing physicians to diagnose early. PCR to detect the gene encoding 56-kDa antigen has been used by most workers. This gene is specific to *O. tsutsugamushi*, and variations in the same are responsible for the antigenic and genetic diversity of *O. tsutsugamushi* (Fournier et al. 2008).

IgM ELISA and nPCR results of the age group of ≥ 20 years showed 7.8% and 8.2%, respectively, indicating the highest risk of ST. Our results also coincided with other national surveillance data from Japan and Thailand, where males were at risk of ST, but in all other countries with reports, females are more at risk (Lee et al. 2006). In South Korea, China, Taiwan, and Thailand, farmers were most at risk (38,183/54,558 – 70% of infections in China from 2006–2014). The specificity was 100% and 97%, whereas the sensitivity was 74% and 82% for nPCR and IgM ELISA, respectively. The IFA is considered the gold standard method for ST diagnosis and is expensive, requiring technical expertise. In addition, it is considered imperfect with its limitations.

Patients negative for ELISA were positive for PCR during the early stage of infection, indicating low sensitivity of ELISA. In contrast, those in the intermediate stage showed positive for both. During the later stage of the disease, ELISA showed high antibody titers. One sample showed PCR amplification, which indicates that the patient had not taken any medication at the time and delayed admission. A study revealed that the mortality rate varies from 1 to 40% if left untreated, depending on the endemic area, patient's condition, and strain virulence of *O. tsutsugamushi* (Min et al. 2018). Novel diagnostic assays cannot be validated against IFA alone but instead against a panel of both serological and antigen detection assays. IgM ELISA has good sensitivity and specificity, ease of operation, and quick results and is also suitable for testing many samples, which may be considered a suitable replacement for the Weil-Felix and IFA tests diagnosis of ST. Of 44 patients with undiagnosed fever, 15 (34%) showed positive IgM ELISA, and a male preponderance was observed, with 34 (77%) cases being positive. However, the availability and cost of ELISA are significant problems in India (Isaac et al. 2004).

O. tsutsugamushi has been reported to have a variety of serotypes, the prevalence of which varies between different endemic areas (Usha et al. 2016). The phylogenetic analysis of the 56-kDa gene showed that Karp-related serotypes were predominant in our samples. Other serotypes were not detected, namely, Boryong, Kato, Gilliamm and Kawasaki. A study from Himachal Pradesh reported Kuroki serotypes by serotype-specific PCR. A study from Korea said that the Boryong serotype is distributed throughout the country except for Cheju Island (Choi et al. 1997). In another study, the Karp serotype was distributed in all Thailand regions, whereas the Kawasaki serotype was found in the southern province of Thailand (Manosroi et al. 2006). The fluctuation and diversification of vector species harboring *O. tsutsugamushi* in local endemic areas may facilitate genetic recombination among diverse genotypes (Kim et al. 2017).

In general, the strength of this study was the large sample size and the use of well-characterized clinical cases. A total of 10,349 clinical samples suspected of scrub typhus were included in the present investigation. Six-year data have confirmed that most acute febrile disease patients were admitted to the hospital between 2014 and 2021. The large sample size allowed us to perform stratified analysis with an adequate statistical number.

The major limitation of this study was the sample size of 10, of which eight were favorable to the Kuroki type, and the other two were nonresponsive to the serotype-specific primers (Bakshi et al. 2007). We did not perform whole genome sequencing for many samples because of limited resource settings. The collection of single serum specimens for detecting IgM antibodies during the acute phase of the disease and a rise in the antibody titer in paired sera could not be detected.

Conclusion

In this study, based on a 56-kDa gene analysis, our serotypes of *O. tsutsugamushi* strains are similar to Karp-related types, improving the understanding of the regional differences between circulating strains and insight into potential resources for future regional diagnostic studies and vaccine development**.** This information would help to understand the evolution of *O. tsutsugamushi* and may help correlate the severity of the disease with serotypes or genotypes during future outbreaks.

Abbreviations ST: Scrub typhus; IgM ELISA: IgM antibody enzyme-linked immunosorbent assay; nPCR: Nested polymerase chain reaction; kDa: Kilodalton; IFA: Immunofluorescence assay; O tsutsugamushi: Orientia tsutsugamushi; WHO: World Health Organization

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Author contribution SD and RKR designed the study. SD performed experiments. SD and SRN analyzed the data; SD wrote the first draft. All authors read and approved the manuscript.

Data availability The data supporting this study's findings are available from reasonable request.

Compliance withethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Ethical approval and consent to participate in this study was approved by the Institutional Ethics Committee.

Informed consent Written informed consent was obtained from each study participant before the interview, sample collection, and testing.

Consent for publication For this type of study, consent for publication is not required.

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