

SHORT COMMUNICATION



# Evaluation of Real Time PCR Detection of the 47 kDa Gene for Reliable Diagnosis of Pediatric Scrub Typhus

Sara Chandy<sup>1</sup>, Sudheer Kumar Peddi<sup>2</sup>, M. Gurumoorthy<sup>1</sup>, Hemalatha Ramachandran<sup>2</sup>, M. S. Ramya<sup>1</sup>, S. Balasubramanian<sup>2</sup> and Anand Manoharan<sup>2,\*</sup>

## Abstract

**Objective:** Diagnosis of scrub typhus is difficult, because of its non-specific clinical features and a lack of reliable serological assays. This study assessed real time PCR detection of the 47 kDa gene along with routine IgM ELISA for confirmatory diagnosis of scrub typhus.

**Methods:** This observational study was conducted between March 2021 and February 2023. Real time PCR detection of the 47 kDa gene was used as a diagnostic assay. Confirmed scrub typhus cases were defined by IgM ELISA and/or real time PCR positivity.

**Results:** A total of 105 children (0–18 years of age) were enrolled, 30 (28.57%) of whom were confirmed to have scrub typhus, according to IgM ELISA and/or real time PCR positivity. The clinical symptoms of scrub typhus positive children included fever, diarrhea, abdominal pain, breathing difficulties, lymphadenopathy, rash, headache, edema, seizure, and poor intake. Good statistical correlations were observed among real time scrub positivity, high grade fever of 8.2 days' duration, the presence of eschar and thrombocytopenia. Non-specific respiratory symptoms; cough, and cold were more severe in patients with scrub typhus. The real time PCR test showed good specificity in discriminating scrub typhus from enteric fever, a common pediatric disease.

**Conclusion:** Detection of the 47 kDa gene with real time PCR has good sensitivity and specificity. The number of days between fever onset and sampling, as well as the administration of antibiotics before sampling, influence the performance of real-time PCR.

**Keywords:** Real-time PCR, 47 kDa, laboratory diagnosis, scrub typhus

## \*Corresponding author:

anandmanoharan@ctmrf.org (AM)

<sup>1</sup>The CHILDS Trust Medical Research Foundation (CTMRF), 12-A, Nageswara Road, Nungambakkam, Chennai 600034, Tamil Nadu, India  
<sup>2</sup>Kanchi Kamakoti CHILDS Trust Hospital, 12-A, Nageswara Road, Nungambakkam, Chennai 600034, Tamil Nadu, India

Received: November 20 2023

Revised: December 22 2023

Accepted: January 25 2024

Published Online: February 24 2024

## INTRODUCTION

*Orientia tsutsugamushi* is an obligate, intracellular, small, Gram-negative zoonotic bacterium causing scrub typhus (ST), an acute febrile disease. ST is transmitted by bites from trombiculid mites (also known as chiggers), and it affects people of all ages [1,2]. Humans are accidental hosts.

In India, ST is responsible for approximately 50% of undifferentiated fever cases during the cooler monsoon and post monsoon months of the year [3-5].

The characteristic clinical manifestations of ST are fever, rash, myalgia, and headache. Eschars, lesions at the bite site, are pathognomic of ST. But their occurrence in patients ranges from 7%–97% of

cases [6]. Doxycycline is the antibiotic of choice, and children typically respond favorably in 1–2 days. Untreated ST can manifest systemically with neurological, gastrointestinal, respiratory, and renal complications [5,6].

Serology (ELISA) remains the test of choice in India, because of its cost, accuracy indices, ease of performance and interpretation [7]. Anti-IgM levels remain above the diagnostic threshold for approximately 1 year after infection, thus hindering differentiation between acute infection and previous exposure when serology is performed on single serum samples [8].

Real time polymerase chain reaction (PCR) has improved the diagnosis of fastidious organisms such as rickettsiae. The 47 kDa gene of *O. tsutsugamushi* is a relatively conserved specific outer membrane protein antigen gene with only 3.3% nucleotide sequence divergence among strains. Molecular testing requires early sampling, ideally before the administration of antibiotics [9–12].

The primary objective of this study was to assess the value of using the 47 kDa gene along with ST IgM ELISA for confirmatory diagnosis of ST.

## MATERIALS AND METHODS

This prospective observational study was performed at a tertiary care pediatric hospital on patients with clinically suspected ST (with fever, with or without eschar, with respiratory and gastrointestinal symptoms). The study was initiated after ethical clearance was obtained from the Institutional Ethical Committee (ref: IEC-DNB/55/May 2021). All participants were enrolled after providing informed consent. Clinical data were recorded on a case report form. Clinical and laboratory data including ST IgM results were obtained from the medical record department of Kanchi Kamakoti CHILDS Trust Hospital. Real time PCR testing was performed at The CHILDS Trust Medical Research Foundation Molecular Laboratory.

### Sample collection

Whole blood ( $n = 95$ ), CSF ( $n = 4$ ), and swabs ( $n = 6$ ) from eschar specimens were collected from eligible participants. All samples were aliquoted on receipt and stored at  $-80^{\circ}\text{C}$  until testing.

### Real time polymerase chain reaction

DNA was extracted from whole blood and CSF with a QIAmp DNA blood mini kit. A QIAmp DNA mini kit was also used for DNA extraction from eschar swabs. To amplify the 47 kDa gene, we performed real time PCR with Takara Premix Ex Taq™ probe qPCR (Takara, Kusatsu, Japan). The human ribonuclease P gene was amplified as an internal control. Positive controls were procured from The Department of Clinical Microbiology, Christian Medical College, Vellore. Real-time PCR was performed with Quant Studio 5 Dx (Thermo Fisher Scientific®). The presence of rickettsial DNA is reported

as the cycle threshold (Ct). The Ct cut-off for detection was  $\leq 35$ .

### Statistical analysis

Data were entered into Microsoft Excel and analyzed in IBM SPSS Statistics version 27. The characteristics of all collected parameters and descriptive statistics (frequency, percentage mean, and standard deviation) are presented. Comparison of categorical data for ST was conducted with chi-square test or Fisher's exact test, as necessary. For continuous data, the comparisons were performed with either Student's t-test or a Mann-Whitney U test. A  $p$ -value  $\leq 0.05$  was considered to indicate statistical significance [13].

## RESULTS

This observational study was performed on 105 pediatric patients. The study participants included 63 (60%) males and 42 (40%) females.

Of the 105 enrolled participants, 30 (28.57%) were diagnosed with confirmed ST, defined by ST IgM ELISA and/or real time PCR positivity, as well as correlating clinical signs and symptoms (Table 1). Of these 30 participants, 20 (66.67%) were males, and 10 (33.33%) were females. Among the PCR positive participants ( $n = 19$ , 18.09%), 11 (57.9%) were males, and 8 (42.1%) were females. The mean Ct value of the ST PCR positive participants was 29.2; 28 (93.3%) of the ST positive participants were  $\leq 10$  years of age, and 19 (63.3%) were  $\leq 5$  years of age; 18 (94.7%) PCR positive ST cases were in children  $\leq 10$  years of age. The highest percentage of confirmed ST cases ( $n = 21$ , 70%) was observed during the cooler and rainy months of the year, from July to December.

Among the PCR positive participants, 18 (94.7%) had fever; 13 (72.21%) had high-grade fever; and 1 (5.3%) had fever with lethargy and intermittent fever. The mean duration of fever in the PCR positive group (8.2 days) was significant ( $p$  value 0.052) compared to that in the PCR negative group. Among the PCR positive participants, 31.6% had cough, and 21.1% had rhinorrhea; the presence of these respiratory symptoms was statistically significant ( $p$  values 0.04 and 0.05, respectively).

The presence of thrombocytopenia (48.1%) and eschar (44.8%) was significantly correlated with confirmed ST positivity ( $p$  value 0.003 and 0.0, respectively). Other

**TABLE 1** | Results of scrub typhus IgM and real time PCR.

Results	Total ( $n = 98$ )	Percentage
IgM+ and PCR+	15	15.31%
IgM+ and PCR-	11	11.22%
IgM- and PCR+	4	4.08%
IgM- and PCR-	68	69.39%
IgM+ and PCR+	30	30.61%

**TABLE 2** | Clinical features of patients with confirmed scrub typhus.

Symptoms and signs <sup>^</sup>	Confirmed scrub typhus** (n = 30)	Others (n = 75)	P value
Fever	29 (96.7%)	71 (97.3%)	1.0
Cough	6 (20.0%)	11 (14.7%)	0.561
Rhinorrhoea	4 (13.3%)	5 (6.7%)	0.272
Diarrhea	3 (10.0%)	16 (21.3%)	0.262
Abdominal pain	2 (6.7%)	7 (9.3%)	1.0
Lymphadenopathy	2 (6.9%)	4 (6.7%)	1.0
Rash	3 (10.7%)	6 (9.5%)	1.0
Headache	2 (6.7%)	2 (2.7%)	0.322
Edema	1 (3.3%)	2 (2.7%)	1.0
Seizure	1 (3.3%)	2 (2.7%)	1.0
Thrombocytopenia	13 (48.1%)	12 (16.7%)	0.003*
Eschar	13 (44.8%)	1 (1.6%)	0.00*

<sup>^</sup>A comparison of clinical features was made for only available patients.

\*A p value less than 0.05 is considered significant.

\*\*Real time PCR and/or ST IgM ELISA positivity.

**TABLE 3** | Real time PCR positivity in the blood, by duration of illness.

Number of days of fever	Real time PCR positive/number of samples for PCR testing
<7 days	4/38 (10.5%)
7–10 days	6/38 (15.7%)
>10 days	7/23 (30.4%)
Total	17/99

Duration of fever was unknown for six outpatients.

clinical symptoms of confirmed ST positive participants included diarrhea, abdominal pain, breathing difficulties, lymphadenopathy, rash, headache, edema, and seizure (Table 2).

The real time PCR showed good specificity (p value 0.021) in differentiating ST from enteric fever, another common pediatric disease. Real time PCR was able to detect ST more than 10 days after onset (Table 3).

One participant died on day 53 of life despite adequate treatment. This patient showed necrotizing pneumonia with pneumatoceles and polymicrobial sepsis, and had a background diagnosis of congenital cystic adenomatoid malformation.

## DISCUSSION

ST is an emerging public health problem in pediatric patients presenting with acute febrile illness. ST is frequently associated with occupational or recreational

activities that facilitate contact of human hosts with chigger vectors. ST is a differential diagnosis for fever with thrombocytopenia. Clinical diagnosis of ST is limited because ST is non-specific and has ambiguous clinical features. Patients often seek healthcare 5–7 days after the onset of illness. Laboratory diagnosis is impeded by samples often being collected approximately 1 week after onset, the use of inaccurate diagnostic tools, and the lack of an early-stage disease detection assay.

ST may manifest non-specifically, or may be associated with clinical features such as fever with chills, headache, body aches, and muscle pain. Several systemic complications are associated with neglected ST, including neurological manifestations; meningitis or meningoencephalitis; and respiratory complications: acute respiratory distress syndrome, and pneumonia. In our study, as previously documented [14–16], high-grade fever was a key clinical feature, along with gastrointestinal and respiratory symptoms. Fever was documented in almost all confirmed ST cases in the present study, in agreement with previous published studies. Thrombocytopenia was a statistically significant finding in the ST cases. One participant with a congenital malformation died despite adequate treatment of necrotizing pneumonia with pneumatoceles and polymicrobial sepsis.

Eschars at the sites of chigger bites have long been considered pathognomic of ST. The eschars usually appear as painless black scabs with an erythematous halo and minimal edema several days after the chigger bite, and before symptomatic manifestation. Eschars have been reported to be observed in 7%–97% cases in endemic areas, and their absence cannot rule out ST [16–19]. Eschars are also non-specific, because they may be observed in gangrene, fungal infections, necrotic bite wounds, and anthrax infections [20]. In this study, eschar was a statistically significant presentation in the confirmed cases.

Serology is the mainstay of diagnosis of ST in tertiary care hospitals in India. Because most patients with ST seek medical care approximately 5 days after the onset of fever, the use of serology analysis is largely advocated. However, false positivity in serology as a result of earlier infections is a major problem. To circumvent false positive findings, ELISA cut-off OD values should be established for each geographic location, to account for background IgM levels.

In the absence of a gold standard indirect immunofluorescence antibody assay for ST, in this study, we defined confirmed ST cases by IgM ELISA and/or real time PCR positivity, as well as correlating clinical features.

Real time PCR detection of the 47 kDa gene has been used to assay rickettsial DNA from whole blood and eschar samples, because of its good sensitivity (97%) and specificity (100%), and the high conservation of the sequence [9]. The lower sensitivity observed in this study might have been due to early use of antibiotics and delays in sample collection after symptom onset. The number of days between disease onset and the collection of whole

blood samples may be an important factor in real-time PCR diagnosis, because a greater number of days is associated with greater chances of IgM secretion, which may obstruct and eradicate the pathogenic organism and prevent it from being detected [20]. In this study, we were able to detect ST even after 10 days of onset.

This study has several limitations. First, the study was conducted at a pediatric multi-specialty hospital, and the results might not represent the actual burden of ST in the community. Most samples for real time PCR were collected more than 5 days after the onset of illness. Theoretically, eschar is the best specimen type for demonstrating rickettsial DNA and often remains positive several days after appropriate treatment has started. In our study, all samples for real time PCR were collected at least 2 days after sample collection for routine IgM testing. Two other gene targets, 56 kDa and groEL, have been recommended for PCR. Use of multiple gene targets can improve the sensitivity of real time PCR [21,22].

Early detection of ST is important to reduce complications and ICU admissions. The 47 kDa gene has performed favorably with respect to other gene targets (56 kDa and groEL) and IgM ELISA, yet it is not used in routine diagnosis [12,23]. This study was conducted to assess the feasibility of using the 47 kDa gene along with IgM ELISA for routine ST diagnostics. On the basis of our findings, clinicians may be advised to use real-time PCR of the 47 kDa gene as an early diagnostic tool to detect ST in antibiotic naive patients. If samples are collected more than 8 days after ST onset, using both real-time PCR and IgM serology to confirm the diagnosis is advisable. However, larger studies are required for evaluation of diagnostic assays for accurate diagnosis of ST, an endemic disease in India.

#### ACKNOWLEDGEMENTS

The authors thank all clinicians for contributing clinical cases. Dr John Jude Anthony Prakash, Professor, Department of Clinical Microbiology, CMC Vellore, is acknowledged for his support in conducting the QC and providing positive controls. Mr. Dipjyoti Dey, Mr. Vignesh M, and Sr Lakshmi B are thanked for technical assistance and data entry. We acknowledge financial support from intramural funds of The CHILDS Trust Medical Research Foundation (CTMRF).

#### CONFLICTS OF INTEREST

There are no conflicts of interest.

#### REFERENCES

- Devasagayam E, Dayanand D, Kundu D, Kamath MS, Kirubakaran R, Varghese GM. The burden of scrub typhus in India: a systematic review. *PLoS Negl Trop Dis*. 2021;15(7):e0009619.
- Mahajan SK, Rolain JM, Kashyap R, Bakshi D, Sharma V, Prasher BS, et al. Scrub typhus in Himalayas. *Emerg Infect Dis*. 2006;12(10):1590-1592.
- Singh S, Patel SS, Sahu C, Ghoshal U. Seroprevalence trends of scrub typhus among the febrile patients of Northern India: a prospective cross-sectional study. *J Family Med Prim Care*. 2021;10(7):2552-2557.
- Varghese GM, Rajagopal VM, Trowbridge P, Purushothaman D, Martin SJ. Kinetics of IgM and IgG antibodies after scrub typhus infection and the clinical implications. *Int J Infect Dis*. 2018;71:53-55.
- Sivaprakasam E, Rajan M, Pasupathy U, Ravichandran L. Clinical characteristics and predictors of severity of pediatric scrub typhus in a tertiary level hospital in South India. *Arch Pediatr Infect Dis*. 2020;8(1):e92752.
- Venkatesgowda PM, Rao SM, Mutkule DP, Rao MV, Taggu AN. Scrub typhus: clinical spectrum and outcome. *Indian J Crit Care Med*. 2015;19(4):208-213.
- Kala D, Gupta S, Nagraik R, Verma V, Thakur A, Kaushal A. Diagnosis of scrub typhus: recent advancements and challenges. *3 Biotech*. 2020;10(9):396.
- Varghese GM, Trowbridge P, Janardhanan J, Thomas K, Peter JV, Mathews P, et al. Clinical profile and improving mortality trend of scrub typhus in South India. *Int J Infect Dis*. 2014;23:39-43.
- Kannan K, John R, Kundu D, Dayanand D, Abhilash KPP, Mathuram AJ, et al. Performance of molecular and serologic tests for the diagnosis of scrub typhus. *PLoS Negl Trop Dis*. 2020;14(11):e0008747.
- Teymouri M, Mollazadeh S, Mortazavi H, Naderi Ghale-Noie Z, Keyvani V, Aghababaei F, et al. Recent advances and challenges of RT-PCR tests for the diagnosis of COVID-19. *Pathol Res Pract*. 2021;221:153443.
- Koh GC, Maude RJ, Paris DH, Newton PN, Blacksell SD. Diagnosis of scrub typhus. *Am J Trop Med Hyg*. 2010;82(3):368-370.
- Kim DM, Park G, Kim HS, Lee JY, Neupane GP, Graves S, et al. Comparison of conventional, nested, and real-time quantitative PCR for diagnosis of scrub typhus. *J Clin Microbiol*. 2011;49(2):607-612.
- MedCalc Statistical Software version 19.2. 6. 2020. (MedCalc Software bv, Ostend, Belgium; <https://www.medcalc.org>).
- Dogra S. Recent advances in understanding pathophysiology of scrub typhus. *JK Science*. 2010;12:70-71.
- Misra UK, Kalita J, Mani VE. Neurological manifestations of scrub typhus. *J Neurol Neurosurg Psychiatry*. 2015;86(7):761-766.
- Saxena A, Khiangte B, Tiewsoh I. Scrub typhus complicated by acute respiratory distress syndrome and multiorgan failure; an unrecognized alarming entity in central India: a report of two cases. *J Family Med Prim Care*. 2014;3(1):80-83.
- Patricia KA, Hoti SL, Kanungo R, Jambulingam P, Shashikala N, Naik AC. Improving the diagnosis of scrub typhus by combining *groEL* based polymerase chain reaction and IgM ELISA. *J Clin Diagn Res*. 2017;11(8):DC27-DC31.
- Prakash JAJ. Scrub typhus: risks, diagnostic issues, and management challenges. *Res Rep Trop Med*. 2017;8:73-83.
- Audhya M, Abirami D, Srikanth S. Atypical eschar: an unusual cutaneous manifestation of scrub typhus. *J Vector Borne Dis*. 2015;52(3):267-269.
- Jim WT, Chiu NC, Chan WT, Ho CS, Chang JH, Huang SY, et al. Clinical manifestations, laboratory findings and complications of pediatric scrub typhus in eastern Taiwan. *Pediatr Neonatol*. 2009;50(3):96-101.
- Paris DH, Blacksell SD, Nawtaisong P, Jenjaroen K, Teerarattkul A, Chierakul W, et al. Diagnostic accuracy of a loop-mediated isothermal PCR assay for detection of *Orientia tsutsugamushi* during acute scrub typhus infection. *PLoS Negl Trop Dis*. 2011;5(9):e1307.
- Anitharaj V, Stephen S, Pratheesh P. Scrub typhus in Puducherry, India: application of nested PCR targeting three different genes - 56 kDa, 47 kDa and groEL of *Orientia tsutsugamushi* and comparison with ST IgM ELISA. *J Vector Borne Dis*. 2020;57(2):147-152.
- Jiang J, Chan TC, Temenak JJ, Dasch GA, Ching WM, Richards AL. Development of a quantitative real-time polymerase chain reaction assay specific for *Orientia tsutsugamushi*. *Am J Trop Med Hyg*. 2004;70(4):351-356.