

# Diagnosis of Rickettsial infection by Multiplex Real-time PCR in Febrile Pediatric Patients Admitted in a Tertiary Care Hospital at Chennai, South India

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## Abstract

Rickettsioses caused by *Rickettsia* and *Orientia* spp. are the re-emerging diseases in India, which are grossly underdiagnosed, particularly among children. They usually present as mild-febrile illness but may extend to severe life-threatening complications. Early diagnosis followed by proper treatment reduces the morbidity and mortality. Non-specific clinical symptoms and lack of point of care diagnosis may delay the treatment. Molecular assays like PCR may helpful in the early diagnosis and confirmation of rickettsial diseases. In this study, we used multiplex real-time PCR to detect *Rickettsia* spp. and *Orientia* spp. in febrile pediatric patients. Whole blood was collected from 239 clinically suspected febrile pediatric patients aged between 6 months to 12 years admitted in tertiary care hospital at Chennai, South India. Multiplex real-time PCR was used to target the *gltA* gene for *Rickettsia* spp. and the *47kDa* gene for *Orientia tsutsugamushi*. To compare the sensitivity, nested PCR was performed on the *56kDa* antigen gene of *O. tsutsugamushi* and the *Rickettsia* genus specific *gltA* gene. By multiplex real-time PCR, 15 samples were positive for *O. tsutsugamushi* and 3 were positive for *Rickettsia* spp. Nested PCR identified 35 positive samples for *O. tsutsugamushi* and 4 positive samples for *Rickettsia* spp. Even though multiplex real-time PCR had lower positivity than nPCR, it was effective in diagnosing *O. tsutsugamushi* and *Rickettsia* spp. in a single assay.

**Keywords:** *Rickettsia*, *Orientia tsutsugamushi*, Real time-PCR, *47kDa* gene, *gltA* Gene, Multiplex PCR, Rickettsial Infection

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## INTRODUCTION

*Rickettsia* and *Orientia* are the group of obligate intracellular gram negative bacteria, belongs to the Rickettsiaceae family. Members of the genus *Rickettsia* and *Orientia* are broadly classified into Spotted fever group, Typhus group and Scrub typhus group Rickettsiae.<sup>1</sup> Rickettsioses caused by *Rickettsia* and *Orientia* are the emerging and re-emerging diseases in India,<sup>2</sup> they are generally under-diagnosed particularly among children.<sup>3-5</sup> Rickettsiae have been found in vectors and small mammals such as rats in their natural environment, while humans become infected through arthropods by accidental contact.<sup>1,6</sup> Rickettsioses often begin with mild self-limiting febrile illness that can progress to severe disease including multi-organ dysfunction and death. Although timely diagnosis and treatment reduce morbidity and mortality, non-specific symptoms similar to other tropical diseases such as dengue, malaria, leptospirosis, and enteric fever, as well as a lack of point-of-care diagnosis, may delay the diagnosis.<sup>2,3,6,7</sup> Serology is the preferred method for diagnosis; however, limitations including the cross-reactivity across *Rickettsia* species, delay in seroconversion (up to two weeks testing) and requirement of acute & convalescent serum for the confirmation of acute infection affect the diseases diagnosis.<sup>1,8-11</sup> Molecular diagnostic tools such as real-time PCR are extremely useful in the early detection of Rickettsial infections from various clinical samples, since they have good sensitivity, a short turnaround time and the ability to target many species in a single assay.<sup>12</sup> Hence in the present study, prevalence of *O. tsutsugamushi* and *Rickettsia* spp. were determined in children with acute febrile illness using multiplex real-time PCR.

## MATERIALS AND METHODS

Pediatric patients aged between 6 months to 12 years, admitted for acute febrile illness at tertiary care center, Chennai, between August 2019 and February 2021 was included in the study. Whole blood was collected from children suspected with Rickettsial infection. Clinical suspicion was determined based on the fever (>3 days but not exceeding 14 days) together with any of the following symptoms

like myalgia, nausea, vomiting, diarrhoea, cough, breathlessness, rash, eschar, lymphadenopathy, hepatomegaly and splenomegaly. Children having fever from causes other than Rickettsial infection (Dengue, Malaria, typhoid and leptospirosis) were excluded from the study. This study was approved by Institute's Ethics Committee (IHEC Approval No: UM/IHEC/F.RM/2021-XI) and Children parents/guardians provided written informed consent to participate in this study.

DNA was extracted using QIAamp DNA blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction. Appropriate positive and negative controls were included in the study. Nested PCR was done to identify *Rickettsia* spp. and *O. tsutsugamushi* using *gltA* (338bp) and the *56kDa* type specific antigen (483 bp) gene specific primers, respectively.<sup>13,14</sup>

In the present study, *Rickettsia* spp. and *O. tsutsugamushi* were identified in a single run using previously published primers and probes (Table 1) of the *gltA* (Citrate synthase) and *47kDa* genes.<sup>15,16</sup> Human IFN- $\beta$  gene was used as an internal control to assess the efficacy of the PCR.<sup>16</sup>

Multiplex real-time PCR was optimised by varying the annealing temperature and the primer concentration in QuantStudio™ 5 real-time thermocycler (Applied Biosystems). The cycling conditions were; initial denaturation of 95°C for 3 minutes followed by 35 cycles of denaturation at 95°C for 10 seconds, annealing at 55°C for 20 seconds and extension at 72°C for 20 seconds. In a 25  $\mu$ L reaction mixture, 12  $\mu$ L of probe master mix (Premix Ex Taq™, TAKARA BIO INC), 10 pmol of each forward & reverse primer and probe (for *Rickettsia* spp. *gltA*, *O. tsutsugamushi* *47kDa* gene), 5pmol of forward & reverse primers and probe (for *hu IFN- gene*) and 5  $\mu$ L template DNA were used.

GraphPad Prism v8.4.3 for Windows was used to perform statistical analysis. The qualitative variables were presented as frequencies (percentage) and quantitative variables as mean, standard deviation (SD)/median and Inter-quartile range (IQR).

## RESULTS

Out of 239 samples, 35 were positive for *56kDa* type specific gene of *O. tsutsugamushi* and

**Table 1.** Details of the primers and probes used

Target gene		Primer Sequence (5' to 3')	Product size	Ref.
47kDa	Forward	CCATCTAATACTGTACTTGAAGCAGTTGA	118bp	(16)
	Reverse	GTCTAAATTCTCATTAAATTCTGGAGT		
	Probe	HEX-TCATTAAGCATAACATTTAACATA CCACGACGA-BHQ1	74bp	(15)
gltA	Forward	TCGCAAATGTTCCACGGTACTTT		
	Reverse	TCGTGCATTCTTTCCATTGTG		
	Probe	FAM-TGCAATAGCAAGAACCGTAGGCTG GATG-BHQ1		
hu IFN $\beta$	Forward	GTCTGCACCTGAAAAGATATTATGG	85bp	(16)
	Reverse	CTGACTATGGTCCAGGCACA		
	Probe	CY5-CTCCTTGGCCTTCAGGTAATGCAG-BHQ3		

**Table 2.** Clinical and Laboratory data of Rickettsioses positive patients

Clinical and Laboratory Profile (n=18)			
Patient Characteristics		Laboratory Data	
Fever duration (days)	6.167 $\pm$ 2.358	Thrombocytopenia	13 (72.22%)
Age	5.667 $\pm$ 3.236	Hyponatraemia	11 (61.11%)
Vomiting	10 (55.55%)	Average Ct Value	
Myalgia	5 (27.77%)	ST 47kDa gene (n=15)	28.66 $\pm$ 2.020 SD
Eschar	12 (66.66%)	Pan Rickettsia gltA gene (n=3)	30.96 $\pm$ 1.662 SD
Lymphadenopathy	6 (33.33%)	hu IFN- $\beta$ gene (n=235)	22.47 $\pm$ 1.185 SD
Hepatomegaly	5 (27.77%)	Multiplex Realtime gltA positive	03
Splenomegaly	4 (22.22%)	Multiplex Realtime 47kDa positive	15

4 were positive for *gltA* gene specific for *Rickettsia* spp. by nested PCR. Among the *56kDa* positive samples 30 with good sequence reads were sequenced and submitted in Genbank (data not shown). However, several attempts were made to sequence the *gltA* gene and we were unsuccessful to get sequence data from four positive samples due to poor DNA quality. Multiplex real-time PCR was performed subsequently to identify Scrub typhus and Rickettsial infection. 4 samples (negative for *gltA* and *56kDa* by nPCR) were excluded from the assay due to low DNA quality. Of the 235 samples analyzed by multiplex real-time-PCR, 15 were positive for *O. tsutsugamushi* and three were positive for *Rickettsia*. Only 14 of the 35 Scrub typhus positive samples confirmed by *56kDa* nPCR (unpublished data) were positive for the *O. tsutsugamushi* 47kDa gene by multiplex real-time PCR. While one ST suspected sample which was negative by *56kDa* nPCR was found to be positive by multiplex real-time PCR. *Rickettsia gltA* gene was observed in three out of 235 samples, compared

to four positive Rickettsial infections detected by *gltA* nPCR. The average Ct (Cycle threshold) value for ST 47kDa gene was 28.66 $\pm$ 2.020 SD (minimum range was 23.7 and maximum 32.0) and 30.96 $\pm$ 1.662 SD for *Rickettsia gltA* gene. The internal control *hu IFN- $\beta$*  (human interferon beta gene) was present in all the 235 samples with average Ct value of 22.47 $\pm$ 1.185 SD (Table 2). Positive control for the *O. tsutsugamushi* 47kDa gene and the *Rickettsia gltA* gene had Ct values of 22.39 and 27.6, respectively. Amplification was absent in the negative control.

The 18 positive children (positive for Scrub typhus and *Rickettsia* by multiplex real-time PCR) showed the following clinical findings: vomiting (55.55%), myalgia (27.77%), eschar (66.66%), lymphadenopathy (33.33%), hepatomegaly (27.77%) and splenomegaly (22.22%). Thrombocytopenia (72.22%) and hyponatremia (61.11%) were the most common laboratory findings. Maculopapular rash was absent in all the 18 patients. The mean duration

of fever at the time of sample collection was  $6.167 \pm 2.358$  and the mean age was  $5.667 \pm 3.236$  (Table 2).

## DISCUSSION

In the present study, 7.7% of children (18/235) showed positive for Rickettsioses using multiplex real-time PCR. Maculopapular rashes were not observed in children in our study, although pathognomonic eschar was seen in 66.66% of rickettsioses positive children. In contrast, in a previous study, they reported absence of both eschar and rash in Rickettsioses positive children.<sup>17</sup> One of the most common clinical symptoms associated with Rickettsial disease in children was hyponatremia.<sup>18</sup> Hyponatremia was found in 61.11% of children with confirmed Rickettsioses. In addition, the most prevalent laboratory finding in positive cases (72.22%) was thrombocytopenia. Rickettsioses has been reported from various regions of India and most of that data was based on serology.<sup>3,5,19-22</sup> Rickettsioses were one of the most common causes of acute febrile disease, contributing significantly to morbidity and mortality in untreated patients. Most diagnostic laboratories in developed countries continue to depend on serological tests to diagnose Rickettsioses.<sup>23</sup> Despite the fact that detectable antibody levels are reached after 7 to 10 days, the confirmation of acute illness in terms of treatment is dependent on both acute and convalescent sera.<sup>8,24</sup> Delays in disease identification and treatment of Rickettsioses result in a poor prognosis, which raises the mortality rate; essentially molecular diagnostic techniques that detect both Rickettsial and Scrub typhus infection at the same time could be useful in disease management.<sup>25,26</sup> The Multiplex method of PCR have been utilized in some of the studies for the identification of *rickettsial* organism, either *Rickettsia* species alone or with combination of *Orientia* spp.<sup>25,26,27</sup> We have successfully identified both *Rickettsia* spp. and *O. tsutsugamushi* with multiplex real-time PCR. Our study was definitely one among the few studies which have utilized the analytical power of multiplex real-time PCR for the identification of *O. tsutsugamushi* and *Rickettsia* spp. in a single assay, which would be helpful for the physician

for the early identification and initiation of anti-rickettsial treatment in advance.

The lower positivity in multiplex real-time PCR compared to nPCR could be due to the quality and quantity of DNA from clinical samples. If multiplex real-time PCR was performed immediately after sample collection, the actual number and sensitivity of the assay might be improved by using other clinical samples such as buffy coat and/or eschar samples.

## CONCLUSION

Rickettsioses caused by *Rickettsia* and *Orientia* spp. are the main causes of febrile illnesses in India. Even though they are easily treatable with available antimicrobial agents, the delay in diagnosis due to non-specific symptoms and lack of point-of-care diagnosis increase the morbidity and mortality. In comparison to serological assays, PCR improves early diagnosis and confirms Rickettsial infections; nevertheless, the high cost of the molecular method limits its usage for routine diagnosis. Our study successfully diagnosed the Rickettsial and Scrub typhus illnesses in a single assay. When compared to single-plex real-time PCR, this assay is substantially more cost-effective.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## AUTHORS' CONTRIBUTION

PD, RM, SK and KS designed the study. RM, SK & KS performed all the experiments. PD supervised the experiments. PD, RM, SK and KS performed data analysis. SK clinically collaborated in the study. RM, SK and KS 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.

## REFERENCES

- La Scola B, Raoult D. Laboratory diagnosis of rickettsioses: current approaches to diagnosis of old and new rickettsial diseases. *J Clin Microbiol.* 1997;35(11):2715-2727. doi: 10.1128/jcm.35.11.2715-2727.1997
- Rathi N, Rathi A. Rickettsial infections: Indian perspective. *Indian Pediatr.* 2010;47(2):157-164. doi: 10.1007/s13312-010-0024-3
- Kalal BS, Puranik P, Nagaraj S, Rego S, Shet A. Scrub typhus and spotted fever among hospitalised children in South India: Clinical profile and serological epidemiology. *Indian J Med Microbiol.* 2016;34(3):293-298. doi: 10.4103/0255-0857.188315
- Khan SA, Dutta P, Khan AM, et al. Re-emergence of scrub typhus in northeast India. *Int J Infect Dis.* 2012;16(12):e889-e890. doi: 10.1016/j.ijid.2012.05.1030
- Kumar S, Aroor S, Kini PG, Mundkur S, Gadiparthi M. Clinical and Laboratory Features of Rickettsial diseases in children in South India. *Pediatr Oncall J.* 2019;16:9-16. doi: 10.7199/ped.oncall.2019.11
- Gunasekaran K, Bal D, Varghese GM. Scrub Typhus and Other Rickettsial Infections. *Indian J Crit Care Med.* 2021;25(2):S138-S143. doi: 10.5005/jp-journals-10071-23841
- Singhi S, Chaudhary D, Varghese GM, et al. Tropical fevers: Management guidelines. *Indian J Crit Care Med.* 2014;18(2):62-69. doi: 10.4103/0972-5229.126074
- Allen L. Richards Worldwide detection and identification of new and old rickettsiae and rickettsial diseases. *FEMS Immunol Med Microbiol.* 2012;64(1):107-110. doi: 10.1111/j.1574-695X.2011.00875.x
- Biggs HM, Behravesh CB, Bradley KK, et al. Diagnosis and Management of Tickborne Rickettsial Diseases: Rocky Mountain Spotted Fever and Other Spotted Fever Group Rickettsioses Ehrlichioses and Anaplasmosis - United States. *MMWR Recomm Rep.* 2016;65(2):1-44. doi: 10.15585/mmwr.rr6502a1
- Hechemy KE, Raoult D, Fox J, Han Y, Elliott LB, Rawlings J. Cross-reaction of immune sera from patients with rickettsial diseases. *J Med Microbiol.* 1989;29(3):199-202. doi: 10.1099/00222615-29-3-199
- Robinson MT, Satjanadumrong J, Hughes T, Stenos J, Blacksell SD. Diagnosis of spotted fever group Rickettsia infections: the Asian perspective. *Epidemiol Infect.* 2019;147:e286. doi: 10.1017/S0950268819001390
- Denison AM, Amin BD, Nicholson WL, Paddock CD. Detection of Rickettsia rickettsii Rickettsia parkeri and Rickettsia akari in skin biopsy specimens using a multiplex real-time polymerase chain reaction assay. *Clin Infect Dis.* 2014;59(5):635-642. doi: 10.1093/cid/ciu358
- Furuya Y, Yoshida Y, Katayama T, Yamamoto S, Kawamura A Jr. Serotype-specific amplification of Rickettsia tsutsugamushi DNA by nested polymerase chain reaction. *J Clin Microbiol.* 1993;31(6):1637-1640. doi: 10.1128/jcm.31.6.1637-1640.1993
- Prakash JA, Sohan Lal T, Rosemol V, et al. Molecular detection and analysis of spotted fever group Rickettsia in patients with fever and rash at a tertiary care centre in Tamil Nadu India. *Pathog. Glob Health.* 2012;106(1):40-45. doi: 10.1179/2047773212Y.0000000001
- Stenos J, Graves SR, Unsworth NB. A highly sensitive and specific real-time PCR assay for the detection of spotted fever and typhus group Rickettsiae. *Am J Trop Med Hyg.* 2005;73(6):1083-1085. doi: 10.4269/ajtmh.2005.73.1083
- Tantibhedhyangkul W, Wongsawat E, Silpasakorn S, et al. Use of Multiplex Real-Time PCR To Diagnose Scrub Typhus. *J Clin Microbiol.* 2017;55(5):1377-1387. doi: 10.1128/JCM.02181-16
- Biswal M, Zaman K, Suri V, et al. Molecular confirmation & characterization of Rickettsia conorii in north India: A report of three cases. *Indian J Med Res.* 2020;151(1):59-64. doi: 10.4103/ijmr.IJMR\_92\_18
- Hullatti C, Latha GS, Veeresh Babu BV. Hyponatremia: a diagnostic marker for the diagnosis of Rickettsial diseases. *Int J Contemp Pediatr.* 2017;4(3):696-699. doi: 10.18203/2349-3291.ijcp20170993
- Mane A, Kamble S, Singh MK, Ratnaparakhi M, Nirmalkar A, Gangakhedkar R. Seroprevalence of spotted fever group and typhus group rickettsiae in individuals with acute febrile illness from Gorakhpur India. *Int J Infect Dis.* 2019;79:195-198. doi: 10.1016/j.ijid.2018.10.024
- Akshatha P, Vijayalakshmi J. Prevalence of Rickettsial infections in a tertiary care centre. *Indian J Microbiol Res.* 2020;7(1):6-9. doi: 10.18231/j.ijmr.2020.002
- Stephen S, Gunasekaran D, Pradeep J, Ambrose S, Sarangapani K. Serological Diagnosis of Indian Tick Typhus in and around Puducherry: Application of Indirect Immunofluorescence Assay. *J Clin Diagn Res.* 2019;13(5):DC06-DC09. doi: 10.7860/JCDR/2019/41743.12887
- Tripathi CDP, Singh M, Agarwal J, Kanta C, Atam V. Seroepidemiology of Spotted Fever Rickettsiosis in Uttar Pradesh: A Prospective Study. *J Clin Diagn Res.* 2017;11(6):DC04-DC09. doi: 10.7860/JCDR/2017/25926.10029
- Kelly DJ, Richards AL, Temenak J, Strickman D,

- Dasch GA. The past and present threat of rickettsial diseases to military medicine and international public health. *Clin Infect Dis.* 2002;34(4):S145-S169. doi: 10.1086/339908
24. Fournier PE, Jensenius M, Laferl H, Vene S, Raoult D. Kinetics of antibody responses in *Rickettsia africae* and *Rickettsia conorii* infections. *Clin Diagn Lab Immunol.* 2002;9(2):324-328. doi: 10.1128/CDLI.9.2.324-328.2002
25. Reller ME, Dumler JS. Optimization and evaluation of multiplex quantitative PCR assay for detection of nucleic acids in human blood samples from patients with spotted fever rickettsiosis, typhus rickettsiosis, scrub typhus, monocytic ehrlichiosis, and granulocytic anaplasmosis. *J Clin Microbiol.* 2020;58(9):e01802-19. doi: 10.1128/JCM.01802-19
26. Peniche-Lara G, Moo-Salazar I, Dzul-Rosado K. A multiplex PCR assay for a differential diagnosis of rickettsiosis, lyme disease and scrub typhus. *J Vector Borne Dis.* 2022;59(2):178-181. doi: 10.4103/0972-9062.337506
27. Paris DH, Blacksell SD, Stenos J, et al. Real-time Multiplex PCR for detection and differentiation of rickettsiae and *Orientia*. *Trans R Soc Trop Med Hyg.* 2008;102(2):186-193. doi: 10.1016/j.trstmh.2007.11.001