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.

Rickettsioses caused by *Rickettsia* and *Orientia* are the emerging and re-emerging diseases in India, they are generally under-diagnosed particularly among children. 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. 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. 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. 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. 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.

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. Human IFN-β gene was used as an internal control to assess the efficacy of the PCR.

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 µL reaction mixture, 12 µL of probe master mix (Premix Ex TaqTM, 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-β* gene) and 5 µL 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
were positive for \textit{gltA} gene specific for \textit{Rickettsia} spp. by nested PCR. Among the \textit{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 \textit{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 \textit{gltA} and \textit{56kDa} by nPCR) were excluded from the assay due to low DNA quality. Of the 235 samples analyzed by multiplex realtime-PCR, 15 were positive for \textit{O. tsutsugamushi} and three were positive for \textit{Rickettsia}. Only 14 of the 35 Scrub typhus positive samples confirmed by \textit{56kDa} nPCR (unpublished data) were positive for the \textit{O. tsutsugamushi 47kDa} gene by multiplex real-time PCR. While one ST suspected sample which was negative by \textit{56kDa} nPCR was found to be positive by multiplex real-time PCR. \textit{Rickettsia gltA} gene was observed in three out of 235 samples, compared to four positive Rickettsial infections detected by \textit{gltA} nPCR. The average Ct (Cycle threshold) value for \textit{ST 47kDa} gene was 28.66±2.020 SD (minimum range was 23.7 and maximum 32.0) and 30.96±1.662 SD for \textit{Rickettsia gltA} gene. The internal control \textit{hu INF-β} (human interferon beta gene) was present in all the 235 samples with average Ct value of 22.47±1.185 SD (Table 2). Positive control for the \textit{O. tsutsugamushi 47kDa} gene and the \textit{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 \textit{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

\begin{table}
\centering
\caption{Details of the primers and probes used}
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\hline
Target gene & Primer Sequence (5’ to 3’) & Product size & Ref. \\
\hline
\textit{47kDa} & Forward & CCATCTAATCTGACTTGAAGCAGTTGA & 118bp (16) \\
& Reverse & GTCCTAAATTCTATTTAATCTGGAGT & \\
& Probe & HEX-TCATTAAGCATAACATTACATA & 74bp (15) \\
\textit{gltA} & Forward & TCGCAATCGTTCAGGCTTTT & \\
& Reverse & TCGTGCATTCTTTCTGATGT & \\
& Probe & FAM-TGCAATACCAACCTTAGGCT & \\
\textit{hu INF-β} & Forward & GTCTGCCAAGTCGAAAGATATTAT & 85bp (16) \\
& Reverse & CTGACTATGGTCAGGCACA & \\
& Probe & CY5-CTCCTTGCCCTTACAGTAATGCAG-BHQ3 & \\
\hline
\end{tabular}
\end{table}

\begin{table}
\centering
\caption{Clinical and Laboratory data of Rickettsioses positive patients}
\begin{tabular}{llll}
\hline
Clinical and Laboratory Profile (n=18) & & Laborator & \\
Patient Characteristics & Laboratory Data & \\
\hline
Fever duration (days) & 6.167±2.358 & Thrombocytopenia & 13 (72.22%) \\
Age & 5.667±3.236 & Hyponatraemia & 11 (61.11%) \\
Vomiting & 10 (55.55%) & Average Ct Value & \\
Myalgia & 5 (27.77%) & \textit{ST 47kDa} gene (n=15) & 28.66±2.020 SD \\
Eschar & 12 (66.66%) & \textit{Pan Rickettsia gltA} gene (n=3) & 30.96±1.662 SD \\
Lymphadenopathy & 6 (33.33%) & \textit{hu INF-β} gene (n=235) & 22.47±1.185 SD \\
Hepatomegaly & 5 (27.77%) & Multiplex Realtime \textit{gltA} positive & 03 \\
Splenomegaly & 4 (22.22%) & Multiplex Realtime \textit{47kDa} positive & 15 \\
\hline
\end{tabular}
\end{table}
of fever at the time of sample collection was 6.167±2.358 and the mean age was 5.667±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.\(^{17}\) One of the most common clinical symptoms associated with Rickettsial disease in children was hyponatremia.\(^{18}\) 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.\(^{3,5,19-22}\) 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.\(^{23}\) 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.\(^{8,24}\) 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.\(^{25,26}\) 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.\(^{25,26,27}\) 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 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.

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