Comparison of Nested Polymerase Chain Reaction and Real-Time Polymerase Chain Reaction Targeting 47kDa Gene for the Diagnosis of Scrub Typhus

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Abstract

Introduction: Scrub typhus is a zoonotic infection caused by Orientia tsutsugamushi which is transmitted by Leptotrombidium mites. The disease manifests as a mild-to-severe illness with non-specific clinical symptoms. Rapid diagnosis and prompt treatment are essential for patient management. Both serological and molecular methods are used for the diagnosis of scrub typhus. The present study assessed the usefulness of detection of the gene encoding the 47kDa outer-membrane protein (OMP) for the laboratory diagnosis of scrub typhus.

Materials and Methods: Nested polymerase chain reaction (nPCR) and real-time PCR targeting 47 kDa OMP antigen gene of O. tsutsugamushi were performed on ethylenediaminetetraacetic acid blood samples. Results: Six of the 103 (5.8%) patients showed the presence of 47kDa gene by nPCR. Seventy of 103 (67.9%) cases showed the presence of 47kDa gene by qPCR. Among the 70 positive cases, the majority of them were females (40/70, 57.1%). The highest number of positive cases was observed during October–February. Conclusion: Real-time PCR targeting O. tsutsugamushi-specific 47-kDa gene is more sensitive than nPCR and may be the assay of choice for the detection of the organism in patients with suspected scrub typhus.

Keywords: 47kDa, nested polymerase chain reaction, real-time polymerase chain reaction, scrub typhus

Introduction

Scrub typhus is caused by Orientia tsutsugamushi and is the most prevalent human rickettsial infection. It affects one billion people in endemic areas and it is the cause of one million infections annually.[1] The disease ranges from mild-to-severe, and mortality rates range between <1% and 50% depending on the time taken to start antibiotic treatment, the immune status of the infected host and characteristics of the strain of Orientia.[2]

This zoonotic infection spreads by the bite of the larval stage of infected Leptotrombidium mites of the family Trombiculidae. The mites feed on human tissue fluids and non-specific symptoms such as fever, breathlessness, myalgia, vomiting and nausea occur 2–10 days after the bite.[3,4] The disease may progress to a severe form with pneumonia, myocarditis, meningoencephalitis, acute renal failure, gastrointestinal bleeding, splenomegaly, hepatomegaly and seizures.[5,6] Rapid diagnosis and prompt treatment are essential for patient management. Serologic tests such as indirect immunofluorescence assay, immunoperoxidase test, enzyme-linked immunosorbent assay, passive hemagglutination test and immunochromatographic assay (rapid card test) are currently available and widely used. However, these serologic tests do not diagnose the disease in the early stages when antibody levels may be low. Polymerase chain reaction (PCR) detection of specific O. tsutsugamushi genes such as 56kDa, 47kDa and 16srRNA has been used for the rapid diagnosis of scrub typhus. The 47-kD gene, which is a specific outer-membrane protein (OMP) antigen gene is relatively conserved, with only 3.3% nucleotide sequence divergence.[7]

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The present study attempts to compare the nested PCR (nPCR) and the real-time PCR targeting 47kDa gene of *O. tsutsugamushi*.

**Materials and Methods**

**Study population**

A total of 103 blood samples were collected during 2014–2018 from patients with PUO in tertiary care hospitals, with symptoms of scrub typhus infection such as fever without an apparent focus of infection and with headache, myalgia, nausea and abdominal discomfort, non-specific rash, lymphadenopathy and eschar. Samples were collected from the Stanley Medical College and Hospital Chennai (44 samples), Employers State Insurance Corporation hospital, Chennai (29 samples), Villupuram Medical College and Hospital, Mundiyampakkam (18 samples), Saveeta Hospital, Chennai (1 sample), Vijaya Hospitals, Chennai (4 samples) and Rajiv Gandhi Government Medical College and Hospital, Chennai (7 samples). The approval for the study was obtained from the Institutional Human Ethics Committee (UM/IHEC I 04-2014-I).

**Specimen collection and processing**

Three millilitres of venous blood was collected, of which 2 ml was in ethylenediaminetetraacetic acid (EDTA) and 1 ml in a plain tube.

**Molecular methods**

**Nucleic acid extraction**

The EDTA blood samples were subjected to DNA extraction using the QIAamp DNA Mini Kit (Qiagen, GmBh, Germany) according to the manufacturer’s instructions. The purified DNA samples were quantified and stored at −20°C.

**Quantification of DNA**

Quantity and quality of the DNA were checked using NanoDrop UV spectrophotometer (Thermo Scientific, USA) at 260 A and 280 A.

**Nested polymerase chain reaction**

nPCR was performed for the detection of the 47kDa OMP antigen gene. The reaction mixture contained 2.5 μl of 10× Taq buffer, 0.8 μl of 10 mM dNTP mix, 1 μl of 10 picomoles Otr263 forward and Otr471133 reverse primers [Table 1], 0.5 μl of 3X Taq polymerase (Bangalore Genei, India), 1 μl of template and volume was made up to 25 μl with MilliQ water. The first round cycling conditions were 95°C for 3 min followed by 29 cycles of 94°C for 30 s, 60°C for 1.5 min and 72°C for 1 min with the final extension at 72°C for 5 min. The second round cycling conditions were 95°C for 3 min followed by 24 cycles of 94°C for 30 s, 64°C for 1 min and 72°C for 1 min with the final extension at 72°C for 5 min. The standardisation was done with the positive controls (Karp and Gilliam) with the PCR product size of 118 bp.[8,9]

Real-time PCR was performed (Thermo Fisher Scientific, 7500 Fast Instrument, Foster City, CA, USA) for the *O. tsutsugamushi* 47kDa and human ribonuclease (RNase) P genes using previously described primers and probes.[8,9] Briefly, the reaction mix for 47kDa and RNase P genes consisted of 12.5 μl of 2X TaqMan Fast advanced master mix (Foster City, CA, USA), 2 μl of (5 picomoles) Otsu630 forward primer and Otsu747 reverse primer and 2 μl of (2 picomoles) OtsuPR655 TaqMan probe and 1 μl of (4 picomoles) RNase P forward and reverse primers, 1 μl of (1 picomole) RNase P probe [Table 1], 5 μl of template and volume adjusted to 25 μl with Milli-Q water for both targets. The cycling conditions for both targets were 95°C for 5 min followed by 45 cycles of 95°C for 30 s and 60°C for 1 min. The results were analysed using 7500 V2.3 software (Thermo Fisher Scientific, Foster City, CA, USA).

**Results**

Six of the 103 (5.8%) patients showed the presence of the 47kDa gene by nPCR [Figure 1]. Seventy of 103 (67.9%) cases showed the presence of 47kDa gene by real-time PCR TaqMan assay. Among the 70 positive cases, the majority of them were females (40/70, 57.1%). The Ct values of all the positive samples ranged from 26.0 to 36.05. The highest number of positive cases was observed during October–February. Of the 103 patients, 21 had eschar formation, and of 70 real-time PCR positive cases, 13 (18.5%) patients had eschar formation. Among six nPCR positive cases, four had eschar formation. Eight patients who developed eschar were negative for both nPCR and qPCR tests [Figure 2].

Of 103 samples included in the present study, 38 (36.8%) were collected during the 1st week of fever (<7 days). Forty-seven (45.6%) cases were collected from patients with 7–10 days of fever. Eighteen cases (17.5%) were collected from patients who had >10 days of fever. Three of the 38 (7.9%) samples were positive by nPCR during the 1st week of fever (<7 days). Three of the 47 (6.4%) cases showed positivity by nPCR when samples were collected between the 7th and 10th day of fever and nPCR failed to detect infection from patients who had >10 days of fever. However, real-time PCR was positive in 28/38 cases (73.68%) during the 1st week of fever, 29/47 cases (61.7%) between the 7th and 10th day of fever and 13/18 cases (72.2%) in patients with >10 days of fever. The association between the duration of fever in the participants and the detection rates of the nPCR and real-time PCR targeting 47 kDa is shown in Table 2.

**Figure 1:** n47kDa positive samples. Lane 1: OTS49, Lane 2: OTS66, Lane 3: OTS83, Lane 4: OTSa 85, Lane 5: OTS 86, Lane 6: OTM96, Lane 7: PC (Karp), Lane 8: Negative control, Lane M: 100 bp DNA ladder.
Scrub typhus is underdiagnosed in India, due to its non-specific presentations, and lack of confirmatory diagnostic tests. Previous studies have reported scrub typhus from many Indian states such as Jammu and Kashmir, Himachal Pradesh, Haryana, Rajasthan, West Bengal, Sikkim, Uttarakhand, Assam, Arunachal Pradesh, Nagaland, Maharashtra, Karnataka, Andhra Pradesh, Kerala, Tamil Nadu and Pondicherry, and may account for up to 50% of undifferentiated febrile illnesses, especially during cooler months. Various research studies showed the emergence and re-emergence of scrub typhus in South India proving the need for awareness and rapid diagnosis of this disease. Scrub typhus responds well to antibiotic therapy, and in patients with a delayed diagnosis, this disease may cause fatal complications. The 47kDa OMP contains both scrub typhus group reactive and strain-specific B-cell epitope. Many studies have documented the usefulness of real-time PCR targeting 47kDa for diagnosing scrub typhus at the early phase. The real-time PCR is as sensitive as nPCR with major advantages of faster results with minimal contamination for the early diagnosis of scrub typhus. The sensitivity ranges from 50% to 83%, whereas the specificity is almost 100%. Eight patients, who were clinically suspected to have scrub typhus, with eschar formation were negative by both the methods (nPCR and real-time PCR). This may be due to low-copy number or due to excess host DNA. PCR of eschar material is considered more sensitive than blood; however, we did not do PCR of eschar material in this study. There are some reports that this may not be useful in a setting where eschar formation itself is not common. Some studies have reported that the presence of eschar formation is highly variable, and occasionally, the eschars may go unnoticed in the dark-skinned people.

Thus, real-time PCR targeting *O. tsutsugamushi*-specific 47kDa gene is more sensitive than nPCR and may be the assay of choice for disease diagnosis in patients with suspected scrub typhus.

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**Table 1: List of primers used in the study**

| Primer            | Sequence 5'-3'                              | Methods                                      |
|-------------------|---------------------------------------------|----------------------------------------------|
| Otr47-263FP       | GTGCTAAGAAARGATGATACCTC                     | Nested PCR first set primers                 |
| Otr47-1133RP      | ACATTTAACATACACCGACGAT                    | Nested PCR second set primers and real-time primers |
| OtuFP 630         | AACTGATTTATTCAAATGCTGCT                    |                                              |
| OtuRP 747         | TAGCCTGATGAAAGATACRTGAATGAATT              |                                              |
| Otu665PR Probe    | FAM-TGGATGACCTTGGTACCCGACGCTTATCT-TAMRA    | Real-time primers                            |
| RNase P FP        | AGATTGGAGCCCTGGAGGGCC                     |                                              |
| RNase P RP        | GACGGCGGCTCTCCACAAAG                     |                                              |
| RNase P Probe     | FAM-TTCTGACCTGAAAGGCTGCGCG-BHQ1          |                                              |

PCR: Polymerase chain reaction

**Table 2: Comparison of nested polymerase chain reaction with real-time PCR for 47kDa**

| Number of days of fever | Number of samples | Nested PCR - 47kDa positive | Real-time 47kDa positive |
|------------------------|-------------------|------------------------------|--------------------------|
| <7                     | 38                | 3                            | 28                       |
| 7-10                   | 47                | 3                            | 29                       |
| >10                    | 18                | 0                            | 13                       |
| Total                  | 103               | 6                            | 70                       |

PCR: Polymerase chain reaction

**Figure 2: Comparison of 47kDa positivity and eschar formation**

negative by nPCR, which may indicate the low sensitivity of the test. Eight patients, who were clinically suspected to have scrub typhus, with eschar formation were negative by both the methods (nPCR and real-time PCR). This may be due to low-copy number or due to excess host DNA. PCR of eschar material is considered more sensitive than blood; however, we did not do PCR of eschar material in this study. There are some reports that this may not be useful in a setting where eschar formation itself is not common. Some studies have reported that the presence of eschar formation is highly variable, and occasionally, the eschars may go unnoticed in the dark-skinned people.
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Conflicts of interest
There are no conflicts of interest.

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