Original Article

Detection of Scrub Typhus by Real-Time Polymerase Chain Reaction and Immunoglobulin M ELISA among Patients with Acute Febrile Illness

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Abstract

Background: Scrub typhus caused by *Orientia tsutsugamushi* is a vector-borne zoonotic infection endemic in several parts of the globe. The infection generally presents with fever and nonspecific clinical features but may lead to severe complications with a high mortality rate if untreated. Early diagnosis and timely management are therefore important. Serological diagnosis such as Weil–Felix test, indirect immunofluorescence assay, immunoglobulin (Ig) M/IgG ELISA, and rapid antibody detection assays are either less sensitive or laborious. Molecular detection by polymerase chain reaction (PCR) targeting specific gene targets of *O. tsutsugamushi* is warranted. **Materials and Methods:** We developed a real-time PCR assay targeting 47-KDa htrA gene for the specific diagnosis of the pathogen. The assay was evaluated in a buffy coat from whole blood or serum samples collected from patients presenting with acute febrile illness. Randomly selected samples were also tested for IgM by commercial IgM ELISA assay. **Results:** The real-time PCR assay was able to detect <1 genome copy per the PCR input and specific to *O. tsutsugamushi* on heterologous pathogens testing. The samples were negative by real-time PCR and 13 samples were positive by IgM ELISA. This study found a relatively low prevalence of scrub typhus in the population. **Conclusion:** The assay developed in this study could be a useful diagnostic tool for the detection of *O. tsutsugamushi* in clinical samples. The study also indicated the need for a wide epidemiological survey that could help determine appropriate health measures including treatment and prevention.

Keywords: Acute febrile illness, *Orientia tsutsugamushi*, real-time polymerase chain reaction, scrub typhus

Introduction

Orientia tsutsugamushi is obligate intracellular bacterium that causes scrub typhus, a serious zoonotic infection transmitted by chigger mites. The infection generally presents as an acute febrile illness along with chills, headache, myalgia, profuse sweating, vomiting, enlarged lymph nodes and sometimes rashes and rarely eschar at the site of the bite.[1] Complications sometimes lead to neurological sequelae, multi-organ failure, and fetal miscarriage or abortion in pregnant women.[2-4] Among the bacterial etiologies of acute febrile illness, scrub typhus is common, especially in tropical and subtropical countries. The infection is now considered important in countries such as Africa, the Middle East, and South America.[3] Scrub typhus is now widespread in both Asia, USA and Australia.[5] It is being seen even in urban areas, and the epidemiology of this infection indicates it to be an important public health problem.[6] The mortality rate is about 13% in untreated cases. Specific and early diagnosis and

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treatment, as well as prevention through immunoprophylaxis, is the approach to control this disease.[7] The serodiagnosis of scrub typhus depends on the less sensitive Weil–Felix test and the immunofluorescence test which is laborious and requires expertise. Immunoglobulin (Ig) M ELISA based on 56-kDa antigen does not detect antigenically variable strains because the antibodies are not cross-reactive.[8-10] Culture for scrub typhus agent in established cell lines such as L929 requires biosafety level-3 and hence is not feasible in a routine laboratory.[11] We developed an in-house real-time PCR for the detection of *O. tsutsugamushi* with high assay sensitivity and specificity. The assay was able to detect down to less than one genome copy per PCR input. We have compared the real-time PCR results with commercial IgM ELISA assay.

Materials and Methods

Patient recruitment and sample collection

A total of 1101 samples were collected from patients presenting with acute febrile illness. The samples were from four centers, namely (1) Sri Narayani Hospital and Research Centre (SNHRC), a 300-bed multispecialty hospital in Vellore district, South India; (2) an upgraded primary health center (PHC) in Ussoor, Vellore district; (3) King Institute of Preventive Medicine (KIPM), Chennai; and (4) Pushpagiri Institute of Medical Sciences (PIMS), a tertiary care hospital in Thiruvalla, Kerala [Table 1].

In this cross-sectional study, samples from SNHRC were collected prospectively from May 2017 to November 2018; samples from PHC were collected between mid-August 2018 and mid-November 2018. KIPM-shared samples were picked randomly from the archives of samples collected between April 2018 and August 2018. Samples collected from PIMS were collected during the monsoon and postfloods of August 2018.

Whole blood samples were collected from the former two centers and serum samples were collected from the latter two centers. Samples were obtained following their consent/assent in English or vernacular language. A detailed clinical pro forma was completed at the time of sample collection. The number of samples collected from each center is shown in Table 1. Ethical approval from the Institutional Review Board (IRB) has been obtained for the study (Independent Ethics Committee/IRB No: 27/16/09/13) and authorization for collecting samples from PHC was obtained from the Directorate of Public Health and Preventive Medicine, Chennai.

Buffy coat was separated from the ethylenediaminetetraacetic acid blood as described previously (Nandagopal *et al*. 2010) for the samples collected at SNHRC and PHC. The buffy coat was suspended in phosphate buffer saline (pH 7.3) and plasma samples were stored at −20°C until use. Serum samples collected from KIPM and PIMS were stored at −80°C until further use.

TaqMan real-time polymerase chain reaction testing

The DNA extraction was performed on the buffy coat and serum using QiaAmp DNA mini kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. An extraction control containing nuclease-free water in the place of the clinical sample was included as every fifth sample. The DNA extractions were carried out in batches and subsequently stored at −20°C immediately until testing.

Primers and probes specific for *O. tsutsugamushi* were designed using the Integrated DNA Technologies (IDT) primer design tool. The *htrA* target gene was used for PCR based on previous literature that reported evaluation in clinical samples with high-accuracy indices. The *O. tsutsugamushi* strain Kato HtrA (htrA) gene encoding 47 kDa protein (NCBI accession No. HM595492; nucleotide position: 457–529) was used as a reference strain for the primer and probe design. The forward primer (5'-CAGTCTAGAGTAGGAGATCAGG-3'), reverse primer (5'-TGCCATTTGTTACTGTTCCTC-3'), and probe (5'-TGCAATAGGTAGTCCTTTCGGT-3') were used for the amplification. Cy5 and BHQ2 were used as a fluorescent dye and a quencher dye, respectively.

The housekeeping gene, GAPDH, acted as an internal amplification control. For the GAPDH gene amplification, forward primer (5'-GGAAACTGTGGCGTGATG-3'), reverse primer (5'-CGTTCAGCTCAGGGATG-3'), and probe (5'-AGTAGAGGCAGGGATGATGTT-3') were used. Texas red and BHQ2 were used as a fluorescent dye and a quencher dye, respectively.

Establishment of the lower limit of detection

Synthetic DNA spanning the target region cloned in a plasmid vector was commercially obtained. The plasmid construct was diluted in serial logarithmic dilutions up to 10^{-12} in TE buffer (pH 8.0). Each dilution was tested in triplicates by the in-house real-time PCR. Negative controls replacing the template with water were used as every third

Table 1: Samples tested from four different study centers

Study centres	Samples collected (*n*)	Nature of sample	Number of IgM positives/random samples tested
Sri Narayani Hospital and Research Centre, Vellore, Tamil Nadu	536	Whole blood	4/143
Upgraded PHC, Ussoor, Vellore, Tamil Nadu	184	Whole blood	ND
King Institute of Preventive Medicine and Research, Chennai, Tamil Nadu	120	Serum	0/27
Pushpagiri Institute of Medical Sciences, Thiruvalla, Kerala	261	Serum	9/192

ND: Not done

sample. The PCR runs were validated only if the controls were satisfactory. Amplification shown in the highest dilution (least concentration) in at least two of the three tubes tested at each dilution was taken as the lower limit of detection as plasmid copies per microliter. The approximate number of plasmid copies/µl of DNA suspension was thus established. The calculation for the plasmid copy number for the pathogens tested was according to the standard methods as described previously.[12] The primers, probes, and gene constructs were custom synthesized from Eurofins (Eurofins Genomics, India).

TaqMan real-time PCR targeting the *htrA* gene was carried out using by QuantiTect Multiplex PCR NoROX kit (Qiagen, Hamburg, Germany). The master mix for each reaction contained 1 × PCR buffer containing dNTPs, Taq polymerase enzyme, forward and reverse primer, (1.5 pmol each) and probe (0.5 pmol) and 10 µl of DNA extract as a template. The thermal conditions for the real-time PCR include 15 min 95°C activation step followed by 50 cycles of denaturation at 94°C for 60 s and annealing/extension at 60°C for 60 s (two-step cycling). A negative control replacing the template with water was included as every third sample. The cutoff for real-time PCR endpoints was determined as amplification within the 35th cycle with a typical sigmoid amplification curve as described previously.[13]

Immunoglobulin M ELISA testing

Random samples (n = 362) were selected from samples (n = 917) collected from three centers namely SNHRC, KIPM, and PIMS. IgM ELISA for the diagnosis of *O. tsutsugamushi* was carried out using a commercial IgM ELISA kit (InBios International Inc., Seattle, Washington, USA). The assays were carried out according to the manufacturer's instructions, and the kit was CE certified. With a cutoff of optical density (OD) 0.5, samples with OD value ≥0.5 were considered as reactive and <0.5 were considered nonreactive. The ELISA assay was performed using Mindray ELISA washer MW-12A and Mindray ELISA reader MR-96A. Positive and negative controls were tested in duplicates, and the test samples were tested in singlets.

Results

Of 1101 samples collected from patients with acute febrile illness, the number from males was higher (n = 645) than females (n = 436). The gender details for twenty patients were not available. The age of the patients ranged from 12 days to 91 years (median, 44). Forty-one percent of the patients had a moderate fever with a temperature between 100°F and 102°F. A high-grade fever with a temperature >102°F was recorded in a few patients (6.2%) and others (51%) had a mild fever (<100°F) at the time of presentation. Majority of them had an undulating fever (88.5%) and a few had prolonged fever (8.5%). Appearance of rashes (erythematous/macular/maculopapular/purpuric) was recorded in 13 (1.2%) patients [Figure 1].

The in-house developed real-time PCR assay showed specific amplification for *O. tsutsugamushi* in the respective channels [Figure 2]. The in-house real-time PCR assay had a detection limit of <1 genome copy per 10 µl of PCR input indicating a high assay sensitivity. In the heterologous testing, the real-time PCR did not amplify other rickettsial species (spotted fever group and typhus group), indicating a high specificity. Amplification within the 40th cycle with at least 0.05 fluorescence intensity and a typical sigmoid amplification curve was determined as the cutoff for real-time PCR endpoint as described previously. Any sample resulting in amplification observed after the 30th cycle was subjected to repeat testing. Of 1101 samples tested, none of the samples were positive for *O. tsutsugamushi*.

Among 362 samples tested for IgM by ELISA, a total of 13 (3.6%) samples were positive. Among the positives, seven were males and six were females with a median age of 48 years.[14] The youngest among the IgM positives was a 13-year-old female from SNHRC and the oldest was a 78-year-old male from PIMS. Among the samples collected from SNHRC (n = 143), four samples (2.8%) were positive, and from PIMS (n = 192), nine samples (4.7%) were positive. The number of IgM positives was plotted against the number of patients recruited during the study period and is shown in Figure 1. Among IgM-positive patients from SNHRC, three had animal contacts (canine) and all four had myalgia and intermittent fever with temperature ranging from 99°F to 101°F. The duration of fever among the four positives ranged from 3 to 7 days. Two patients had a high total white blood cell (WBC) count. Two were from a rural area and two others were from the peri-urban area of Vellore district.

Discussion

An in-house real-time PCR targeting the htrA gene of *O. tsutsugamushi* was developed and evaluated in patients with acute febrile illness. Randomly selected samples were tested by IgM ELISA for the determination of IgM status among the patients. Scrub typhus as one of the major causes of acute febrile illness has been reported from many parts of India. A recent study from North India reported IgM seroprevalence of 17% in patients with febrile illness[15] and 4.2% in the general population in South India.[16] In most of the health-care settings, particularly where laboratory facilities are limited, a provisional diagnosis is made followed by empirical treatment with often unnecessary antimicrobials. This substantially reduces the detection limit in the blood sample resulting in misdiagnosis and delayed specific treatment and further complications.

Scrub typhus presents with fever and nonspecific clinical presentation and therefore is difficult to distinguish from other illnesses. The serological techniques such as Weil–Felix test, indirect immunofluorescence antibody assay, IgM ELISA, and culture techniques have many limitations. A molecular diagnostic approach such as PCR offers hope with good sensitivity and specificity.

O. tsutsugamushi being an intracellular pathogen, the detectable number of the pathogens in the blood is very low,

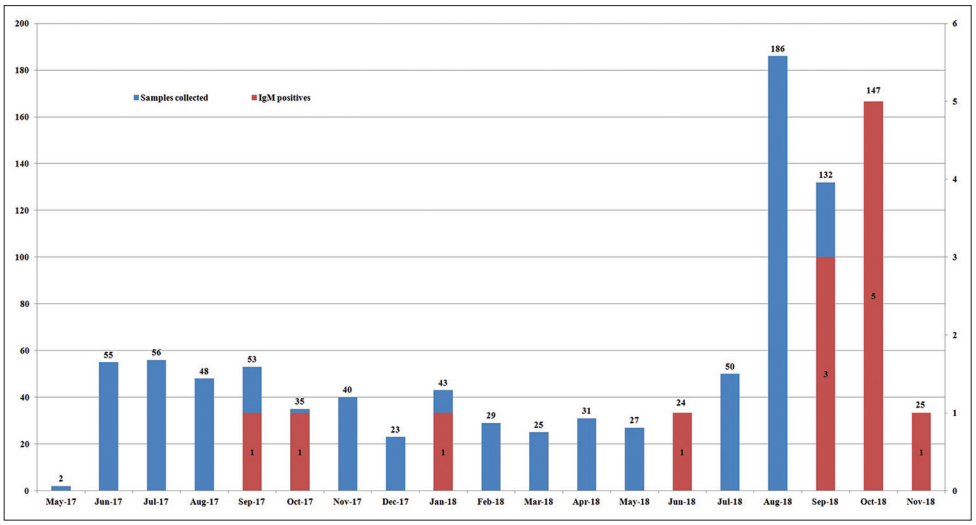

Figure 1: Month-wise distribution of acute febrile illness cases and number of immunoglobulin M positives

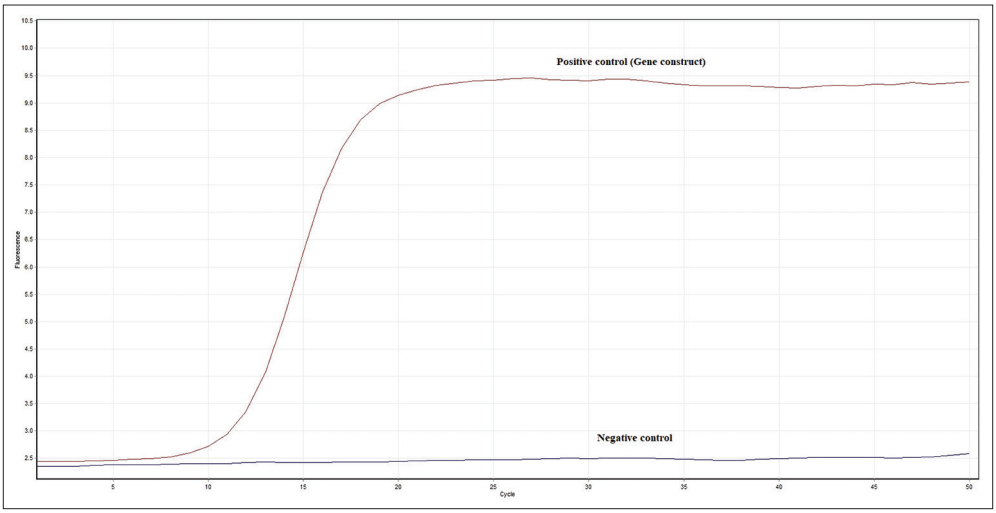

Figure 2 : Real-time polymerase chain reaction amplification for Orientia tsutsugamushi

and blood samples may become a valued specimen only during the bacteremic phase. In a study by Kramme *et al*.,[17] bacteremia was at its peak concentration after 9–12 days of time of infection. Eschar samples are preferred more than blood,[18] and the presence of rickettsial pathogens in eschar and rash samples was not found to be affected by prior use of antimicrobials,[19] but these presentations are not commonly seen in infected patients. The buffy coat has been shown to be an appropriate sample for detection with high sensitivity compared to blood samples,[20] especially when eschar was absent. In our study, eschar was observed in three patients and rashes were observed in 14 patients among the 536 patients recruited from SNHRC whose clinical details were available. The patients with either eschar or rashes were, however, negative for both real-time PCR and IgM ELISA. Eschar samples were not collected which might have influenced the chances of pathogen identification. The frequency of rashes in patients with scrub typhus varies between areas, and in India, it is reported to be 1.7%. Moreover, it is generally difficult to spot rashes in patients with dark skin and some may not present rashes at the time of clinicophysical examination.[21] In our study, we used both buffy coat and serum samples for the identification of *O. tsutsugamushi* by the in-house real-time PCR assay. None of the samples were positive, indicating a very low prevalence of the infections in this population. IgM status among the patients was also low (3.6%) that corroborates with the real-time PCR results. In contrast to previous studies, we found a very low prevalence (3.6%) of scrub typhus IgM in acute febrile illness cases. In general, a maximum number of cases occur during monsoon and post-monsoon season. The low percentage of exposure in this study could be attributed to reduced exposure to vegetation, relative humidity, rainfall, and air pressure (Xu *et al*. 2017).

A total of 13 patients were found positive for IgM among the 362 random samples tested, of which 143 samples were from SNHRC and 192 samples were from PIMS. Three of the four patients positive for IgM reported close contact with dogs.

All four had myalgia and intermittent fever with temperature ranging from 99°F to 101°F. Two patients had a high total WBC count. Two were from rural areas and two others were from the peri-urban areas of Vellore district. Dogs being susceptible to *O. tsutsugamushi* infection, their role in spreading the pathogen in the endemic areas of Asia is investigated.[22] All the four patients had an acute fever and reported within 7 days of illness indicating the usefulness of acute-phase samples for the diagnosis of infection.

Several target regions for the molecular detection of *O. tsutsugamushi* have been reported and these include outer membrane protein A,[23] 56-kDa protein,[24] autotransporter domain proteins,[25] and molecular chaperones GroEL.[26] The target region htrA coding for 47 KDa was chosen based on a thorough literature survey and bioinformatic analysis. The most conserved region was chosen for amplification and is therefore considered highly specific to *O. tsutsugamushi*. In our study, we did not perform an additional PCR assay targeting other regions. The 47 KDa htrA protein has also been shown to be a preferred target region for the specific diagnosis of *O. tsutsugamushi* with high sensitivity and specificity.[27] This protein is also reported to be highly conserved among the strains identified from different countries.[28] We used 47 KDa htrA gene as the target region for the real-time PCR assay. The detection limit was <1 genome copy per PCR input indicating very high sensitivity. The PCR did not amplify other pathogens including typhus group and spotted fever group of rickettsial species indicating a very high specificity.

Serological diagnosis of scrub typhus using IgM ELISA has been shown to be a promising alternative for indirect immunofluorescence assay with comparatively high sensitivity.[29] IgM-based serological approaches need to document baseline titers in a healthy population of that region along with the evaluation of both positive and negative sera panel. This is difficult in most health-care settings, even in places where the disease is endemic. In our study, we used a cutoff of 0.5 based on the previous baseline titer determination.[30] However, it is important to be cautious while making a clinical interpretation based on IgM tests alone. The IgM remains elevated up to 12 months following infection,[31] and use of a single serum sample may give indiscriminate results. Paired samples collected from acute and convalescent phase with the four-fold rise in titer should be used for the diagnosis.[32] In our study, we did not test paired samples, and therefore, the results of IgM ELISA were not conclusive. Hence, it is imperative to test paired sera or confirm by more sensitive molecular tests such as real-time PCR. The other limitation of this study is noninclusion of laboratory-confirmed and clinically suspected cases of acute scrub typhus. The study was intended to report on the prevalence of scrub typhus in patients with acute febrile illness comparing an in-house real-time PCR and commercial IgM ELISA. The study implicated that there is a low frequency of scrub typhus in the population tested, and the real-time PCR assay also corroborates with the findings.

The samples collected in our study were negative by real-time PCR suggesting either the low prevalence of the pathogen in the population or that the samples were collected during the late phase of infection or the prior use of antimicrobials.

Early and specific diagnosis is important for the clinical management of the patients presenting with nonspecific febrile illness. The in-house real-time PCR assay could be a suitable tool for the detection of *O. tsutsugamushi* in clinical samples. For a definitive diagnosis, it is imperative to use appropriate specimens collected during the acute phase of infection and the use of the proper diagnostic tool.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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