Validation of Geno-Sen’s Scrub Typhus Real Time Polymerase Chain Reaction Kit by its Comparison with a Serological ELISA Test

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Abstract
Background: In the recent past, scrub typhus (ST) has been reported from different parts of India, based on Weil-Felix/enzyme-linked immunosorbent assay (ELISA)/indirect immunofluorescence assay (IFA). Molecular tests are applied only by a few researchers. Aims: Evaluation of a new commercial real time polymerase chain reaction (PCR) kit for molecular diagnosis of ST by comparing it with the commonly used IgM ELISA is our aim. Settings and Design: ST has been reported all over India including Puducherry and surrounding Tamil Nadu and identified as endemic for ST. This study was designed to correlate antibody detection by IgM ELISA and Orientia tsutsugamushi DNA in real time PCR. Materials and Methods: ST IgM ELISA (InBios Inc., USA) was carried out for 170 consecutive patients who presented with the symptoms of acute ST during 11 months (November, 2015–September, 2016). All 77 of these patients with IgM ELISA positivity and 49 of 93 IgM ELISA negative patients were subjected to real time PCR (Geno-Sen’s ST real time PCR, Himachal Pradesh, India). Statistical Analysis: Statistical analysis for clinical and laboratory results was performed using IBM SPSS Statistics 17 for Windows (SPSS Inc., Chicago, USA). Chi-square test with Yates correction (Fisher’s test) was employed for a small number of samples. Results and Conclusion: Among 77 suspected cases of acute ST with IgM ELISA positivity and 49 IgM negative patients, 42 and 7 were positive, respectively, for O. tsutsugamushi 56-kDa type-specific gene in real time PCR kit. Until ST IFA, the gold standard diagnostic test, is properly validated in India, diagnosis of acute ST will depend on both ELISA and quantitative PCR.

Keywords: Geno-sen’s scrub typhus polymerase chain reaction, Orientia tsutsugamushi, scrub typhus

INTRODUCTION

Scrub typhus (ST) caused by Orientia tsutsugamushi and transmitted by the chigger mites was once considered a disease of the shrub jungle/war and confined to “Tsutsugamushi triangle.”[1] Now ST is spreading across the world with more endemic foci being identified in India, where ST is an emerging infectious disease and reported from almost every state.[5-25] A few minor ST epidemics have occurred in Southern India, with the diagnosis mostly based on the serological evidence along with clinical presentation.[2-6,8-15,18,20,23-25] Application of molecular techniques for diagnosing ST has been reported by very few Indian researchers in the recent past.[7,16,19,21,22] In this study, we compare ST patients’ IgM antibody response in enzyme-linked immunosorbent assay (ELISA) and presence of O. tsutsugamushi 56-kDa type-specific gene in real time polymerase chain reaction (PCR) quantitative PCR (qPCR), using a commercial kit, which has become available in India only recently and being validated for the first time. The relevance of Weil-Felix (WF) test, particularly OXK agglutination, a nonspecific serological test for ST diagnosis has been critically analyzed.[5,18,26] The main objective of

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this research work is to validate the performance of a new commercial real time PCR kit by comparing this molecular test with the presently available ST IgM ELISA for the diagnosis of acute ST.

**Materials and Methods**

This cross-sectional study was carried out from November 2015 to September 2016 in a tertiary care super specialty teaching hospital Puducherry, South India, after obtaining approval from the Institutional Human Ethics Committee.

**Sample collection**

A total of 170 consecutive patients presenting clinical symptoms of acute ST during this period were included on the basis of predetermined inclusion and exclusion criteria.[18] Among these patients, the first group of 89 were those with ≤7 days fever (3–7 days) and the 2nd group of 81 with >7 days fever (8–25 days). After obtaining written informed consent from the patients, blood was collected in two containers: (a) plain tube without anticoagulant (2–3 ml) and (b) Ethylenediamine tetraacetic acid (EDTA) tube (4–5 ml blood). Serum was separated, aliquoted, and kept frozen at −20°C for ST IgM ELISA. From EDTA tube, buffy coat layer was separated and genomic DNA was extracted using QIAamp DNA Blood Mini Kit as per the manufacturers’ instructions. Extracted DNA samples were kept frozen at −80°C till the time of testing.

**Enzyme linked immunosorbent assay analysis**

For ST conventional ELISA test, ST Detect ELISA IgM (InBios International, Seattle, USA) was used. The procedure followed was strictly in compliance with the instructions provided in the kit.[13,18]

**Molecular diagnosis**

ST qPCR was performed for 126 acute sera collected during the study. All 77 which were positive in ST IgM ELISA were examined. Regarding ST IgM ELISA negative cases, only 49 of 93 samples which had sufficient quantity were subjected to ST qPCR. We have used Geno-Sen’s ST (Rotor Gene) real time PCR kit, Genome Diagnostics Pvt. Ltd, Solan, Himachal Pradesh, India. The primer details are as follows:

- Accession no: KP334159.1
- OtsuF: 5’-AATTGCTAGTGCAATGTCTG-3’,
- OtsuR: 5’-GGCATTATAGTGCTAGGCTGAG-3’,
- www.genomediagnostics.co.in.

The kit targets 56 kDa Type Specific gene of *O. tsutsugamushi*. The master mix contains reagents and enzymes for the specific amplification of the target gene through FAM Channel. Real time PCR was carried out in CFX96 C1000 Touch machine (Bio-Rad, USA). For each test, the reaction mix : R1 – ST super mix 12 µl, R2 - Mg sol - 2.5 µl, R3 – Internal control - 0.5 µl and extracted DNA - 10 µl. Thus, the total volume was 25 µl for each sample. One positive control (10^4 copies of *O. tsutsugamushi* DNA) and one negative control provided in the kit were included in each run. Samples were tested in duplicate. The thermal cycling conditions were: holding temperature 95°C for 10 min, denaturation 95°C for 15 s, annealing 55°C for 20 s for 45 cycles, and extension at 72°C for 15 s. The cycle threshold (Ct) values below 35 were considered positive.

**Statistical analysis**

Clinical and laboratory results were performed using IBM SPSS Statistics 17 for Windows (SPSS Inc., Chicago, USA). Chi-square test with Yates correction (Fisher’s exact test) was employed for small number of samples.

**Results**

Among 170 patients screened for ST during the study by ST IgM ELISA, 77 patients were positive. Of these 77 antibody positive serum samples, only 42 showed evidence for 56-kDa type-specific gene in Geno-Sen’s ST real time PCR assay. Regarding ST IgM ELISA negative samples, 49 of 93 samples which had sufficient quantity were subjected to ST qPCR and seven of them were found to be positive. Ct values ranged from 21.7 to 34.7. Table 1 shows ST IgM ELISA and ST qPCR positivity with reference to duration of febrile illness. Table 2 analyses clinical, hematological parameters, and qPCR results of patients with ST IgM ELISA positivity. The youngest patient with ST IgM antibody positivity was 1-year-old and the eldest patient was aged 78 years. Mean age was 32.13 ± 22.35.

All patients responded well to treatment with doxycycline (adults) and azithromycin (children). Defervescence of fever occurred within 24–48 h of antibiotic therapy.

**Discussion**

WF test is a nonspecific test, but still in use in the developing countries including India, with different cut-off values for OXK agglutinins ranging from ≥1:80 to 1:320 as suggestive of ST.[4,6,8,10,13,14,18,21] However, WF test must be interpreted with caution and in correlation with clinical judgment. For the serological confirmation of ST, indirect immunofluorescence assay (IFA) is the “gold standard.”[17,20] In India, application of ST IFA has been reported only by few[2,5,20,24] and kit is yet to be fully evaluated. In view of the technical complexity and subjectivity of the reporters’ observation, IgM ELISA has been recommended as a viable alternative to IFA by different researchers. InBios ST IgM ELISA has been evaluated by several researchers and found to have good sensitivity and specificity.[9,11,15,18,20,22,27]

**Cross reaction of scrub typhus IgM enzyme linked immunosorbent assay InBios kit with nonscrub typhus diseases**

Recently, Blacksell *et al.* reported 84% sensitivity and 98% specificity of ST InBios ELISA against the gold standard IFA.[27] This kit has been validated by us and found to be quite satisfactory.[13,18,25] Prakash *et al.* observed false positive reactions in patients with falciparum malaria, pulmonary...
A modification to the second criteria has been suggested by Lim et al.,[29] according to whom an initial or convalescent IgM IFA titer of ≥1:3200 is recommended.

For most of the laboratories in the developing world, the first criteria, namely, the isolation of *O. tsutsugamushi* is beyond their reach, due to stringent requirement of bio-safety level-3 containments. Criterion three cannot be applied for patients who provide only single (acute) serum samples. The gene most commonly targeted in ST PCR is 56-kDa.

The commercial kit, Geno-Sen’s ST real time PCR kit, targets this gene. According to the kit manufacturers (Geno-Sen’s ST real time PCR, Genome Diagnostics) specificity is 100%. The kit’s technical brochure mentions that Gene sequence analysis of the amplified region of ST shows a pronounced homology among the various ST subtypes, and no homology with other DNA. RNA and DNA from different viruses and bacteria did not show any positive signal with this kit.

### Table 1: ST IgM enzyme-linked immunosorbent assay and ST qPCR positivity with reference to duration of febrile illness

| Days of fever (same as the sample collection day) | ST IgM ELISA positive (n=77) | ST IgM ELISA negative (n=49*) |
|-------------------------------------------------|-----------------------------|-------------------------------|
| qPCR positive | qPCR negative | qPCR positive | qPCR negative |
| ≤7 days (n=71) | 29 | 19 | 4 | 19 |
| ≥7 days (n=55) | 13 | 16 | 3 | 23 |
| Total (n=126) | 42 | 35 | 7 | 42 |

*Out of 93 ST IgM ELISA negative sera, only 49 were available for PCR. ELISA: Enzyme-linked immunosorbent assay, qPCR: Quantitative polymerase chain reaction.

### Table 2: ST IgM versus quantitative polymerase chain reaction: Clinical and laboratory parameters of patients (n=84*)

| Clinical/Laboratory findings | qPCR positive (n=49) | qPCR negative (n=35) | Total (n=84), n (%) | P^* |
|-----------------------------|----------------------|----------------------|---------------------|----|
| Fever |                     |                      |                     |     |
| ≤7 days (3-7)^1 | 33^1 | 19 | 52 (61.9) | 0.224257 |
| ≥7 days (8-25)^1 | 16^1 | 16 | 32 (38.09) | 0.224257 |
| Chills and rigor | 38 | 26 | 64 (76.19) | 0.729034 |
| Myalgia | 32 | 19 | 51 (60.17) | 0.307921 |
| Headache | 38 | 21 | 59 (70.23) | 0.082827 |
| Cough and expectoration | 40 | 19 | 59 (70.23) | 0.082827 |
| Abdominal pain | 34 | 16 | 50 (59.52) | 0.00688 |
| Hepatomegaly | 17 | 9 | 26 (30.95) | 0.029312 |
| Splenomegaly | 22 | 10 | 32 (38.09) | 0.380127 |
| Nausea | 29 | 12 | 41 (48.80) | 0.128735 |
| Vomiting | 28 | 18 | 46 (54.76) | 0.024407 |
| Pneumonitis | 9 | 6 | 15 (17.85) | 0.603931 |
| Eschar | 19 | 4 | 23 (27.38) | 0.885135 |
| Rash | 8 | 2 | 10 (11.90) | 0.005587 |
| Lymphadenopathy | 12 | 2 | 14 (16.66) | 0.022822 |
| Pedal edema | 3 | 0 | 3 (3.57) | 0.136037 |
| Thrombocytopenia (<1.5 lakhs) | 19 | 5 | 24 (28.57) | 0.014306 |
| Increased liver enzymes (AST/ALP/AP) | 6 | 4 | 10 (11.90) | 0.909318 |
| Creatinine (>1.0) | 5 | 2 | 2 (2.38) | 0.462941 |
| Leukocytosis (>11,000 cumm) | 6 | 2 | 15 (17.85) | 0.128735 |

*Includes 77 ST IgM ELISA positive and 7 ST IgM ELISA negative patients, ^P<0.05 were considered significant, ^Includes 4 ST IgM ELISA negative cases, †Includes 3 ST IgM ELISA negative cases, ‡Same as the sample collection day.

**Clinical/Laboratory findings**

- Fever
- Headache
- Myalgia
- Cough and expectoration
- Abdominal pain
- Hepatomegaly
- Splenomegaly
- Nausea
- Vomiting
- Pneumonitis
- Eschar
- Rash
- Lymphadenopathy
- Pedal edema
- Thrombocytopenia (<1.5 lakhs)
- Increased liver enzymes (AST/ALP/AP)
- Creatinine (>1.0)
- Leukocytosis (>11,000 cumm)

**Clinical/Laboratory findings**

| Test | Phosphatase | ELISA | qPCR |
|------|-------------|-------|------|
| ASI  | ALT         | ALP   |      |

**Clinical/Laboratory findings**

- Fever
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There is a paucity of data regarding genotypes prevalent in India.[19,21,22] At present, only four genotypes are included in the IFA kits: Kato, Karp, Gilliam, and Boryong. Local strains as well as prevalent genotypes in India need to be incorporated in IFA kits, which might indicate the true prevalence of ST and specifically identify more number of positive ST patients. The latest algorithm for diagnosing ST, namely, STIC (Scrub Typhus Improved Criteria) mandates the fulfillment of one or more of the following four options:[28]

1. Isolation of *O. tsutsugamushi* in culture
2. Admission IgM (IFA) titer of ≥1:12,800
3. Four-fold rise in IgM (IFA) titer in paired sample
4. Positivity in PCR targeting any two of the three genes (56-kDa, 47-kDa and groEL).

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A modification to the second criteria has been suggested by Lim et al.,[29] according to whom an initial or convalescent IgM IFA titer of ≥1:3200 is recommended.

For most of the laboratories in the developing world, the first criteria, namely, the isolation of *O. tsutsugamushi* is beyond their reach, due to stringent requirement of bio-safety level-3 containments. Criterion three cannot be applied for patients who provide only single (acute) serum samples. The gene most commonly targeted in ST PCR is 56-kDa.

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Although qPCR was negative for 35 out of our 77 IgM ELISA positive patients, these cannot be totally ignored as false positive ELISA cases, since the detection of 56 kDa gene depends on the duration of febrile illness at the time of blood collection. Normally, ST-qPCR is positive in the 1st week of the acute illness, whereas IgM antibody starts appearing from day 7 onward. According to Saisongkol et al.,[30] ST-PCR positivity was observed up to day 22 in some patients. In 16 of 77 of our IgM ELISA positive but ST-qPCR negative patients, blood was collected between 8 and 25 days of fever. Incidentally, four of our PCR negative patients had typical ST eschar. Significant number of patients with IgM antibody and/or O. tsutsugamushi DNA positivity presented with rash, vomiting, abdominal pain, lymphadenopathy, hepatomegaly, and thrombocytopenia. Twenty-three among 77 IgM ELISA positive patients developed typical ST eschar. It is possible that some cases could have been missed due to the dark skin of the patients and difficulty in conducting a thorough physical examination of the covered parts, due to the social customs prevailing in this country.

The following are the limitations of the study: we could not carry out ST IgM IFA. ST qPCR targeting other two genes, namely, 47-kDa and groEL gene was not performed. Paired serum samples analysis was not performed due to patients’ poor compliance in coming forward voluntarily to give convalescent samples, due to their practical difficulties like traveling from 30 to 200 km.

CONCLUSION
The performance of the commercial qPCR kit is satisfactory. Molecular diagnosis should be carried out preferably during the 1st week of febrile illness. Due to the reasonable levels of sensitivity and specificity of ST IgM ELISA kit, its continued usage in developing countries like India may be unavoidable until ST IFA kits are properly validated and become easily available and affordable. As per the recommendation of Paris et al.,[28] use of both PCR and serology would identify more number of ST cases.

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Conflict of interest
There are no conflicts of interest.

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