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# Screening of Scrub Typhus among Patients Presenting with Pyrexia of Unknown Origin in Tertiary Care Hospital

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**Abstract**
*Scrub typhus is an endemic disease in South-east Asia and is the most important cause among undifferentiated fevers accounting for about 20-35%. Diagnosis of scrub typhus is a challenge, especially in patients with dengue fever, leptospirosis, rickettsioses, typhoid and malaria as they can present with similar signs and symptoms. Serological tests like rapid immunochromatographic tests and Weil-Felix tests are easy to perform and inexpensive, but lack specificity and sensitivity. The main aim of the study is to screen for scrub typhus by using routine serological tests, ELISA and real time PCR. A total of 32 blood samples suspected to be scrub typhus, were tested for serological assays like rapid immunochromatography, Weil-Felix and ELISA, and real time PCR for identifying 47KDa gene specific for Orienta tsutsugamushi Karp strain. A total of 100 samples were considered, which were collected from patients with fever of unknown origin. A total of 68 samples were found to be positive for various infections ruled out by routine microbiological investigations. Scrub typhus screening was done for the remaining 32, out them 12 (37.5%) samples were positive by rapid immunochromatographic test, 9 (28.1%) samples were tested positive by Weil-Felix agglutination test and 7 (21.8%) samples were positive by ELISA. All the 32 samples were selected for performing real-time polymerase chain reaction (RT-PCR), out of these 20 were positive by real-time PCR, which included the 12 samples positive by rapid immunochromatography method.*
**Keywords**: *Weil-Felix, RT- PCR, 47KDa gene, ELISA.*

## Introduction

Scrub typhus is a disease of global importance, it is endemic in areas extending from Northern Japan to Eastern Russia in north, to the territories around the Solomon Dea into Northern Australia in the South and to Pakistan and Afghanistan in the west; this geographical region is known as “tsutsugamushi triangle”. In Asian countries over one million new cases were identified annually and about one billion people are at a risk of developing this scrub typhus[1]. In Indian Scenario several cases were reported from states like Haryana, Jammu & Kashmir, Himachal Pradesh, Uttaranchal, West Bengal, Assam, Maharashtra, Kerala and Tamil Nadu[2]. The incidence of Scrub typhus is increasing during the past several years and persists during cooler months in southern states presenting with fever and myalgia[3].

Scrub typhus is a zoonotic disease caused by *Orienta tsutsugamushi*, which was first observed in

Japan[4]. It clinically presents as acute febrile illness for 7-10 days after the bite of an infected larva of the trombiculid mite (chigger). Arthropod association is stable in case of tick and mite borne Rickettsia infections due to transovarial transmission from female to progeny. *Orienta tsutsugamushi* differs from Rickettsia both genetically as well as morphologically in cell wall composition as it lacks lipopolysaccharide[5]. The word typhus has been derived from Greek word “typhos” for fever and “stupor” meaning smoke or dupa. Scrub typhus an acute febrile illness presents with non-specific signs and symptoms and one of the cause for fever of unknown origin[6]. Sporadic cases due to Rickettsia are prevalent throughout the year and usually go undiagnosed, due to non-specific clinical presentation, less access to specific diagnostic facilities and low index of suspicion by physicians. As far as diagnosis is concerned rapid solid immunochromatographic assays which qualitatively detect IgG, and IgM or IgA antibodies to *Orienta tsutsugamushi* in human blood are available. The rapid tests help in preliminary diagnosis and confirmation can be made by IFA (reference test) and ELISA. Though rapid tests and Weil-Felix tube agglutination methods are easy to perform and inexpensive, they lack specificity and sensitivity. Awareness should be created about the limitations of serological tests and the significance of confirmatory methods like ELISA and PCR must be increased in case of fever of unknown origin.

Almost 30 serotypes of *Orienta tsutsugamushi* which are antigenically distinct co-exist in endemic areas and only one among them may predominate. A high degree of antigenic heterogeneity is seen among the different strains like Karp, Kato and Gilliam, long lasting immunity is seen with homologous than heterologous strains. These strains also vary in their degree of virulence[5]. *Orienta tsutsugamushi* expresses type specific proteins coded by 56KDa and 47KDa genes. The 56KDa gene is unique not expressed by other Rickettsia, so used for vaccine development[7]. The 47KDa gene which is also known as HtrA encodes for the outer membrane protein, is more sensitive by real time quantitative PCR rather than conventional PCR, as the amplicon containment is easier to achieve[7]. This 47KDa gene of Karp strain is highly conserve product, which is both group reactive and species specific, also recognised by both antibodies and ‘T’ cells, they play a significant role in molecular diagnosis.

The present study was done in a tertiary care hospital, here several cases with fever are attended in outpatient department, wards and referred from elsewhere. The samples of blood were collected from patients with fever, initially all the blood samples were subjected for routine microbiological investigations. Those negative by routine methods were screened for scrub typhus by serological methods, followed by real time PCR.

## Materials and Methods

The study was done for a period of one year from December 2014 to November 2015, a total of 100 blood samples were collected under aseptic conditions using rapid plain vacutainer without anticoagulants. The samples were tested initially by routine serological methods and those which were negative selected for scrub typhus screening by ONE STEP scrub typhus antibody test, Weil-Felix test, ELISA, followed by PCR.

**1. One step scrub typhus antibody test:** The SD BIOLINE tsutsugamushi strip test which was precoated with lines, one for the test as ‘T’ (*Orienta tsutsugamushi* antibody test line), and “C” (control line), on the surface was used. The serum sample was added to the strip, the appearance of purple coloured “T” line indicates the presence of IgG/IgA or IgM antibody along with the control line.

**2. Weil-Felix test**: Smooth killed stained antigen suspension was used, which when mixed with serum sample will form agglutination. The antigens were obtained from Government King Institute, Guindy, Chennai. The serum sample is initially diluted as follows 1:20,1:40,1:80,1:160,1:320 and1:640 respectively, after adding the antigen the titres more than 1:160 are considered reactive.

**3. IgG ELISA**: It is based on membrane immunoassay for detection of IgG antibodies in the

serum, the microtiter wells were coated with unique recombinant antigen mix. The serum sample was diluted with the given sample diluent and added to each well. The wells were incubated, washed and treated with IgG enzyme conjugate (HRP) and substrate (TMB) added after second step of incubation and washing. After adding the stopping solution, the absorbance was measured at 450 nm which was directly proportional to the concentration of IgG antibodies. To check the integrity of kit components, a set of positive and negative controls were set up. The results were interpreted with spectrophotometric reading, a value more than cut off was considered reactive and less as non-reactive. The values near the cut off are doubtful and the assay was repeated in triplicate.

**4. Real-Time PCR:** In our study Pure fast DNA purification kit (PURE FAST® Genomic DNA Minispin purification kit), along with HELINI scrub typhus real -time PCR kit from HELINI BIOMOLECUELS, Chennai, India were used. The probe PCR master mix which contains 1.5 U of Hotstart Taq DNA polymerase, 10X Taq reaction buffer, 35mM MgCl2, 1µl of 10 mm dNTPs mix and PCR additives were used. The DNA from the sample was extracted by using the above kit. The purified DNA was stored at -20ºC, the DNA was loaded in the PCR vials, centrifuged and placed into thermal cycles. About 10 µl of nuclease free water instead of purified DNA sample, was added as negative control and 10 µl of positive control were set up.

| | Step | Time | Temp |
|---|---|---|---|
| | Taq enzyme activation | 15min | 95ºC |
| 50 cycles | Denaturation | 20sec | 95ºC |
| | Annealing/Data collection* | 20sec | 58ºC |
| | Extension | 45sec | 72ºC |

**Fig 1:** The thermal profile was set up as follows

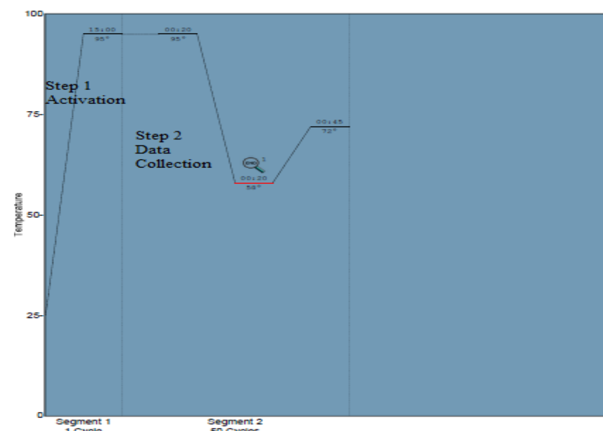


**Fig 2**: Thermal Profile estimated runtime

*Thermal Profile Summary*

| Segment | Cycles | Plateau | Temp. (degrees) | Temp. Inc. (deg/sec) | Duration (min:sec) | Time Inc. (min:sec) | Collect |
|---|---|---|---|---|---|---|---|
| 1 | 1 | Plateau 1 | 95.0 | 0.0 | 15:00 | 00:00 | <none> |
| 2 | 50 | Plateau 1 | 95.0 | 0.0 | 00:20 | 00:00 | <none> |
| 2 | 50 | Plateau 2 | 58.0 | 0.0 | 00:20 | 00:00 | 1 Endpoints |
| 2 | 50 | Plateau 3 | 72.0 | 0.0 | 00:45 | 00:00 | <none> |

Replicates: Treated individually (since no replicates in selection)
* Fluorescence term used: dR

*Text Report*

| Well | Well Name | Well Type | Threshold* | Ct* |
|---|---|---|---|---|
| A6 | NTC | NTC | 211.680 | No Ct |
| B6 | Ecoli DNA | Unknown | 211.680 | No Ct |
| C6 | H1 | Unknown | 211.680 | 46.22 |
| D6 | H2 | Unknown | 211.680 | 47.13 |
| E6 | H3 | Unknown | 211.680 | 44.16 |
| F6 | H4 | Unknown | 211.680 | 44.58 |
| G6 | H5 | Unknown | 211.680 | 44.69 |
| H6 | QS2 | NPC | 211.680 | 25.01 |

**Fig 3:** Thermal profile summary

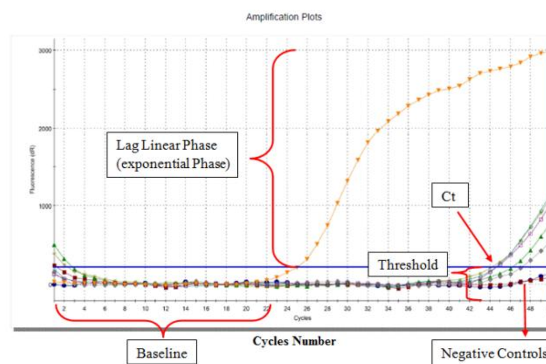


**Fig 4**: Amplification plot (For 1000-fold dilution series)

**Interpretation of real time PCR:** The nucleic acid levels were qualified basing on the raw data analysis, baseline and threshold values which were set on the amplification plot. The plot shows increase in fluorescence between the initial and later plots indicating higher amount of starting templates than the later samples. The fluorescence signal was designated as dR, which was plotted on 'y' axis. The baseline reprsents the background noise level

before a significant amplification occurs (3 to 5 cycles). The threshold indicates the stage at which significant and specific amplification occurs, an exponential phase (linear doubling phase) of amplification and also above the highest baseline values.

## Results

**Table No. 1:** No. of samples collected for scrub typhus from patients with the history of fever.

| Total No. of samples collected | No. of samples positive for scrub typhus | No. of samples positive by various microbiological investigations to rule out fever | No.of samples Negative by various microbiological investigations |
|---|---|---|---|
| 100 | 12 (12%) | 68 (68%) | 20 (20%) |

**Table No. 2:** No. of samples positive for scrub typhus basing on the criteria like fever with eschar and without eschar.

| Criteria | Scrub typhus positive by various methods |
|---|---|
| 1) Fever with eschar | 2 (17%) |
| 2) Fever without eschar | 10 (83%) |
| 3) Total | 12 (100%) |

**Table No.3:** No. of cases positive by three methods out of 32 samples suspected for scrub typhus.

| Sl. No. | Method | Positive |
|---|---|---|
| 1. | Rapid Immunochromatographic test | 12 (37.50%) |
| 2. | Weil-Felix Agglutination test | 9 (28.12%) |
| 3. | ELISA | 7 (21.87%) |

**Table No. 4:** Comparison of Scrub Typhus positive samples by all the four methods.

| Sl. no | Method | Positive |
|---|---|---|
| 1. | Rapid Immunochromatographic test | 12 (100%) |
| 2. | Weil-Felix agglutination test | 9 (75%) |
| 3. | ELISA | 7 (58.3) |
| 4. | Real- time PCR | 12 (100%) |

## Discussion

Scrub typhus a killer disease, can cause disseminated vasculitis and perivascular inflammatory lesions resulting in end organ injury. Even though the disease shows dramatic response to appropriate antibiotics serious complications can occur and most of them prove to be fatal[8]. Scrub typhus an important cause of acute undifferentiated febrile illness in India. It goes undiagnosed, due to nonspecific clinical presentation, limited awareness, low index clinical suspicion & lack of diagnostic facilities[9]. Nonspecific clinical manifestations of fevers among intensive care patients, usually are misdiagnosed and left untreated. So relevant laboratory diagnostic methods associated with specific treatment protocols and supportive management can help in prevention of serious complications like sepsis and multiorgan failure in these patients[8]. According to a study done by Salam Kenny Singh et al., a total of six patients with undiagnosed fever by routine investigations showed positivity for antibody against scrub typhus by rapid immunochromatographic assay[10].

In our study out of 32 samples which were undiagnosed by routine investigations, 12 cases were positive for scrub typhus by rapid immunochromatographic test. A comparative analysis was done by Dong min Kim et al., to study the sensitivity and specificity of conventional PCR (C-PCR), nested PCR (N-PCR) and real time quantitative PCR (Q-PCR) by targeting the *Orienta tsutsugamushi* specific 47kDa gene with blood samples obtained from patients within 4 weeks of onset of fever[7]. He concluded that RT-PCR had 100% sensitivity and specificity and was considered as one of the preferred assays for diagnosis of scrub typhus. In the present study, all the 12 cases that were tested positive for scrub typhus by real time PCR were also detected positive by immunochromatographic test. Another study done recently by M. Vivekanandan et al., mentioned that out of 50 cases positive for scrub typhus, 39 (78%) showed positive by Weil-Felix agglutination test [11]. This report was found to be consistent with our study in which out of 12 positive samples screened by Immunochromatographic test and RT PCR, only 9 (75%) samples were found positive by Weil-Felix test.

In a study made by Kardeshwar P.S. Narvencar et al. out of the 44 patients included in his study, 15 (34%) were found to be positive for IgM antibodies against *Orienta tsutsugamushi*[12]. In our study, 7(58.3%) cases out of 12 positive cases showed positivity for IgM antibodies against *Orienta tsutsugamushi* by ELISA method. This was not found to be as

sensitive as real time PCR and immunochromatographic methods. The major drawback with ELISA was that it was more of a qualitative test than quantitative, the results often depend on the duration of illness and the time of sample collection[13]. The other reason for ELISA giving poor results is mainly, due to the lack of enough seroprevalence studies in India and the discrepancy of antibody cut off titres by the manufacturers of ELISA kits[14]. In our study, among the four diagnostic methods employed for the diagnosis of scrub typhus, Immunochromatographic test and RT PCR remains to be equally reliable methods. Rapid immunochromatographic test were much simple to perform, interpret and economical for screening large number of patients presenting with fever.

Now a days highly sensitive and specific rapid methods are available, and their usage plays an important role in our country as scrub typhus is one of the re-emerging disease especially in North-east and South India. Real time PCR which was considered in our study detects diverse antigenic type making it more specific and sensitive moreover PCR detects during bacteraemia stage even before the antibody response overcoming the problem of high background titres in endemic areas[14]. All currently available serological tests for scrub typhus have limitations. The clinician should be aware of this despite their wide spread use. As per the study by Jitendra Kumar et al. scrub typhus is prevalent in India and there exists a resurgence of disease, although definite statistical data are not available[15]. In his study 49 cases were scrub typhus positive, among which 7 cases presented with meningitis which was confirmed by CSF ADA levels and only 1 case was presented with eschar. In our study among the 12 cases tested positive for scrub typhus, only two cases presented with eschar. So, we can conclude that it is not always the patients with scrub typhus positive should present with eschar, it can manifest with other signs and symptoms. In a serological study for rickettsia infections in Tamil Nadu made by Kumarasu K et al. in December 2005, out of 964 patients, 89 (9.2%) were positive for scrub typhus[16]. In our study, during December 2014 to November 2015 the incidence is slightly increased to 12 (12%) out of 100 samples collected from suspected cases. So, measures need to be implemented to increase awareness, to diagnose and treat this infection in the affected areas.

## Conclusion

Scrub typhus is an emerging disease which is difficult to diagnosis, especially in underdeveloped and developing countries due its nonspecific clinical manifestations, limited awareness and low index of suspicion among clinician including lack of diagnostic facilities. Hence early diagnosis and treatment are imperative to reduce the mortality and complications associated with the disease. Since these organisms disseminate from the skin to target organs, suggesting that they may enter the peripheral circulation. Hence direct detection of organisms in peripheral smears by other serological methods like direct immuno- fluorescence and immuno-alkaline phosphatase methods may help in rapid diagnosis of scrub typhus. Thus, both molecular and serological methods remain as reliable diagnostic methods for diagnosing scrub typhus. Since scrub typhus has become more prevalent in many parts of the country focus has to be made on vaccine development by identifying proper immunodominant antigens and their combinations, which would bring about long-term protection against infections from the different strains of *Orienta tsutsugamushi*.

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