Comparative Evaluation of Serological and Molecular Methods for the Diagnosis of Scrub Typhus in Indian Settings

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SUMMARY: The major brunt of scrub typhus is borne by developing countries like India but the disease remains neglected. The rapid progression of this infection to serious complications and associated mortality calls for improved diagnostics. The immunoglobulin (Ig)M immunofluorescence assay (IFA), with all of its limitations remains the gold standard. Of 218 suspected cases from patients enrolled in a 2-year study, 30 cases of scrub typhus were detected by IgM IFA, using a 1:64 dilution. The sensitivities of the IgM enzyme-linked immunosorbent assay, IgM rapid flow assay (RFA), and IgG RFA were found to be 97%, 87%, and 77%, respectively. Their respective specificities were 100%, 100%, and 92%, respectively. The sensitivity and specificity of a nested PCR assay targeting a gene encoding a 56-kDa protein were found to be 50% and 100%, respectively.

Scrub typhus is a major cause of febrile illness in India. However, even with its overwhelming incidence, it has not received much attention as a public health problem. The rapid progression of the disease and associated mortality calls for immediate and improved diagnostics. Clinical diagnosis of scrub typhus is very difficult because of its indefinite presenting features. The presence of an eschar is pathognomonic but low occurrence of this symptom has been reported in most Indian studies (1–3). An immunofluorescence assay is the diagnostic modality of choice but it suffers from several limitations, including subjectivity, need for technical expertise, and requirement for expensive instruments (4,5). There is a scarcity of literature from Indian settings, reporting on the evaluation of alternate diagnostic methods for routine use.

A prospective study was conducted in our tertiary care center from October 2013 to October 2015. All patients with acute febrile illness presenting fewer than 15 days of fever were enrolled in the study. The study was performed after receiving permission from the Institute's Ethics Committee. Serum and whole blood samples from all patients were collected according to standard methods after receiving informed consent.

The serum samples were subjected to the following tests: i) indirect immunofluorescence assay (IFA; Fuller Laboratories, Fullerton, CA, USA): IFA slides, pre-coated with 4 strains (Gilliam, Karp, Kato, and Boryong), were used. Patient sera were diluted at 1:64 as recommended by the associated literature. This titer was used as the screening cut-off, and end point titers of 1:512 were calculated. The antigen-antibody reaction was visualized using a fluorescent microscope, where a positive reaction was characterized by the visualization of small green fluorescent forms, either intracellular or extracellular, in the background of counterstained red cells; ii) immunoglobulin (Ig)M enzyme-linked immunosorbent assay (ELISA; InBios, Seattle, WA, USA): a qualitative indirect ELISA that detects IgM antibodies to Orientia tsutsugamushi in serum was performed using all samples. The absorbance, which is directly proportional to the number of IgM antibodies present, was measured at 450 nm. The cut-off for IgM ELISA was calculated using samples from 38 healthy volunteers belonging to the same geographical region in which the study was conducted. The cut-off was calculated as mean optical density (OD)₄₅₀ (0.35) + 3 SD (0.18) = 0.89; iii) rapid flow assay (RFP) for IgM and IgG (InBios): a qualitative, membrane based flow assay for the detection of IgM and IgG was performed using all serum samples. The antibodies in the serum sample react with the conjugate (anti-human IgM/IgG colloidal gold) on the sample pad, and the mixture then migrates upwards by capillary action to react with scrub typhus derived recombinant antigen.

The whole blood samples were also subjected to an in-house nested PCR assay. DNA was extracted from 200 µl of whole blood using the QIAamp Blood Mini Kit (Qiagen, Hilden, Germany). DNA amplification was performed using a nested format with conditions described by Furuya et al. (6). DNA from a known case of scrub typhus was used as a positive control. The purified products from 3 random positive samples were sequenced by the Sanger dyeoxy method on an ABI 3730xl automated sequencer. The 95% confidential interval (CI) was calculated using Stata ver. 12.1 (StataCorp, College Station, TX, USA).

A total of 218 suspected cases of scrub typhus with patients presenting with fever for fewer 15 days were analyzed during the study period. A total of 30 cases (14%) of scrub typhus were detected by IgM IFA at a 1:64 dilution. Twelve cases remained positive up to a
dilution of 1:512. Fifteen cases (6.9%) were detected by PCR. Three random PCR-positive samples were confirmed by Sanger sequencing method to be positive for *O. tsutsugamushi*. Their sequences were submitted to the National Center for Biotechnology Information GenBank (KT966737, KT966738, and KT966739).

The sensitivities of IgM ELISA, IgM RFA, and IgG RFA were determined to be 97% (95% CI, 83–100%), 87% (95% CI, 69–96%), and 77% (95% CI, 58–90%), respectively. Their specificities were 100% (95% CI, 88–100%), 100% (95% CI, 88–100%), and 92% (95% CI, 88–100%), respectively. The sensitivity and specificity of nested PCR was found to be 50% (95% CI, 31–69%) and 100% (95% CI, 88–100%), respectively.

IgM ELISA appears to be a good alternative method for serological diagnosis with sensitivity and specificity comparable to IFA. It has no subjectivity issues and does not need technical expertise and expensive instruments such as fluorescent microscopes. However, it is necessary to determine geographically-relevant cut-offs using healthy volunteers.

IgM RFA is a point of care diagnostic test that can be used in field settings. IgM RFA has been shown to be highly specific for diagnosis; however, but variable sensitivities ranging from 34% to 96% have been reported in other studies (7,8). In developing and resource limited settings, IgM RFA appears to be a very valuable test. IgG RFA was slightly less sensitive and specific compared with IgM RFA, based on our study. During infection, IgG antibody appears later and persists longer than IgM, therefore. IgG-based assays can miss very early cases and falsely detect past infections (9).

Most laboratories in India lack a proper molecular set-up; therefore, PCR assays are most often available in research settings only. The PCR assay used in our study showed high specificity but suffered from poor sensitivity, which might further restrict its routine use (10,11). A plausible reason for poor sensitivity could be the initiation of specific therapy in most cases before sending the sample for diagnosis (12,13). Therefore, a high index of suspicion and the availability of pre-treatment samples is of paramount importance when using PCR-based assays as the sole diagnostic modality. In addition, the unavailability of PCR in most Indian laboratories restricts its routine use. False positives obtained by IgM IFA have been reported in some recent studies and thus, it is possible that the poor sensitivity of our PCR assay was reflective of problems with IFA and not the PCR assay.

In conclusion, IgM ELISA and IgM RFA are viable alternatives in resource limited settings, in which early diagnosis and prompt treatment can be potentially life-saving.

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**Conflict of interest** None to declare.

**REFERENCES**