Determination of Cutoff of ELISA and Immunofluorescence Assay for Scrub Typhus

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ABSTRACT

Background: The most common method employed for diagnosis of scrub typhus is serology. It is widely known that demonstration of ≥4-fold rise in titers of antibody in paired sera is required for diagnosis. However, for guidance of initial treatment, there is a need for rapid diagnosis at the time of admission. Therefore, there is a need for standardized region specific cutoff titers at the time of admission. **Materials and Methods:** A total of 258 patients of all age groups with clinically suspected scrub typhus over a period of 24 months (October 2013-October 2015) were enrolled. Serum samples of these patients were subjected to immunofluorescent antibody (IFA) for immunoglobulin M (IgM) (Fuller Labs, USA) with dilutions of 1:64, 1:128, 1:256, and 1:512. Serum samples of all 258 patients were subjected to IgM ELISA (Inbios Inc., USA). Any patient with response to antibiotics within 48 h accompanied by either presence of an eschar or positivity by polymerase chain reaction was taken as positive. Receiver operating characteristic (ROC) curve was drawn to generate cutoff for these tests. **Results:** A total of 20 patients were diagnosed as cases of scrub typhus. The ROC curve analysis revealed a cutoff optical density value of 0.87 with sensitivity and specificity of 100% and 94.12%, respectively. ROC curve analysis of IFA revealed sensitivity and specificity of 100% and 93.5%, respectively at 1:64 dilution. **Conclusion:** Considering cost constraints, centers in and around New Delhi region can use the cutoffs we determined for the diagnosis of scrub typhus.

Key words: Cutoff, ELISA, Immunofluorescent antibody, New Delhi, scrub typhus

INTRODUCTION

Scrub typhus, a neglected zoonotic disease, is a known cause of febrile illness in New Delhi and surrounding regions.[1] It is difficult to distinguish scrub typhus from other co-endemic illnesses based on clinical features alone. Diagnosis, therefore, relies heavily on laboratory tests. The most common method employed for diagnosis of scrub typhus is serology.[2] Immunofluorescence assay is the gold standard, but the most commonly employed method for the diagnosis in most centers in India is Immunoglobulin M (IgM) ELISA.[2,3] It is widely known that demonstration of ≥4-fold rise in titers of antibody in paired sera is required for diagnosis.[4] However, for guidance of initial treatment, there is a need for rapid diagnosis at the time of admission. One of the biggest challenges in the diagnosis of scrub typhus in this region is the unavailability of a standardized cutoff for these serological tests.

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Aim

The aim of the study was therefore to determine geographically relevant cutoffs for ELISA and polymerase chain reaction (PCR).

Settings and design

A prospective, diagnostic study was conducted, whereby a total of 258 patients of all age groups with clinically suspected scrub typhus over a period of 24 months (October 2013-October 2015) were enrolled in the study

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after taking a proper consent. The study was conducted at a tertiary care hospital in New Delhi.

MATERIALS AND METHODS

Serum and whole blood samples were collected from all the patients using standard precautions. The serum samples from patients were subjected to IgM immunofluorescent antibody (IFA) (Fuller Laboratories, USA). The IFA slides were precoated with four scrub typhus strains (Gilliam, Karp, Kato, and Boryong) propagated in L292 cells. Serum samples of all the patients were put at dilutions of 1:64, 1:128, 1:256, and 1:512. The antigen-antibody reaction was visualized using a fluorescent microscope. A positive reaction was visualized as small green fluorescent rods in the background of counterstained red cells. An indirect ELISA that detects IgM antibodies to *Orientia tsutsugamushi* was performed in all serum samples. Serum samples from forty healthy controls were also subjected to IgM ELISA. The absorbance was measured at 450 nm. An in-house nested PCR assay was done on the whole blood sample. DNA was extracted from 200 μl of whole blood using the QIAmp blood mini kit (Qiagen GmBh, Germany). DNA amplification was performed using a nested format using conditions described by Furuya *et al*.[5] For both PCR runs, the amplification protocol consisted of denaturation of template at 94°C for 30 s, annealing at 55°C for 2 min followed by extension at 70°C for 2 min for 35 cycles. External primers p34 and p55 were used to amplify a 1003-bp segment of the *O. tsutsugamushi*-specific 56-kDa gene, and internal primers p10 and p11 were used to amplify a final product of 483-bp segment. DNA from a known case of scrub typhus was used as a positive control. All patients were thoroughly examined for the presence of an eschar. The time taken for the fever to resolve after the initiation of specific antibiotic therapy was recorded. Patients were classified as positive and negative based on the following criteria: Any patient with response to antibiotics within 48 h accompanied by either presence of an eschar or positivity by PCR was taken as positive. A receiver operating characteristic (ROC) curve was drawn each for ELISA and IFA to generate cutoff for these tests. The cutoff for ELISA was also determined using recommendations from kit literature. It was calculated by adding three standard deviations to the mean optical density (OD) value of ELISA runs on the samples of healthy volunteers.

Statistical analysis used

ROC curves were generated using Medcalc Version 12.7 (MedCalcSoftware, Ostend, Belgium). 95% confidence intervals were calculated for sensitivity and specificity of different cutoffs.

RESULTS

A total of twenty patients were diagnosed as cases of scrub typhus. Fifteen of these cases were positive by PCR while an eschar was seen in a total of seven cases. Only two cases with eschar were positive by PCR assay. A set of 40 sera samples collected from healthy volunteers from New Delhi were used to establish region specific cutoff for IgM ELISA. The cut off calculated from healthy volunteer was mean OD (0.35) + 3 standard deviation (0.18) = 0.89. The ROC curve analysis of 258 patients revealed a cutoff-OD value of 0.87 with sensitivity and specificity of 100% and 94.12%, respectively. ROC curve analysis of IFA performed on 258 patient revealed sensitivity and specificity of 100% and 93.5%, respectively at 1:64 dilution. The dilution at 1:512 gave a specificity of around 98%.

DISCUSSION

IFA for scrub typhus, like every other serological test, requires a demonstration of rise in titer in paired sample at least 2 weeks apart. The rapid progression of the disease makes it difficult to wait for 2 weeks before giving the final result. The result in such a scenario is often retrospective. Besides, in most cases, patients are not available for the paired samples. In such a case, there is a need for region specific standardized cutoff titers on admission. Several studies have used different cutoff titers ranging from 1:10 to 1:3200, but there is no consensus.[6] The studies from India have used cutoff titers of 1:64 or 1:128, but a clear, systematic evaluation of cutoff is lacking.[7-9] The Fuller laboratory kit literature recommends a titer of 1:64 as the screening cutoff and determination of endpoint titers in positive cases. The high cost of test restricts determination of endpoint titers in each and every positive case. There is a need for determination of standardized cutoff for New Delhi region which can also be used in other centers of North India. We found that the titer of 1:64 has good sensitivity and specificity on ROC curve analysis. Recent studies have pointed out the imperfect nature of IFA citing the possibility of false positives due to cross-reactivity.[10] In such cases, a titer of 1:512 can be used for diagnosis owing to the high specificity observed at this titer.

ELISA is a comparatively cheaper and easier test to perform without requirement of expensive fluorescent microscopes. Most centers in India use ELISA for the diagnosis. The recently published ICMR guidelines recommend a cutoff-OD value of 0.5.[3] Using uniform cutoff values all across the nation can be a problematic as many of our healthy controls had OD values above 0.5. A wide geographical variation has been noticed in different studies and therefore, a nationalized cutoff OD cannot be determined. Several

studies from India have used a cutoff-OD from 0.5 to 1.[7] We propose a cutoff-OD value of 0.87 or 0.89 for New Delhi and surrounding region based on our findings.

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Conflicts of interest

There are no conflicts of interest.

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