RESEARCH PAPER

Detection of Immunoglobulin M and Immunoglobulin G Antibodies Against *Orientia tsutsugamushi* for Scrub Typhus Diagnosis and Serosurvey in Endemic Regions

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Received: June 11, 2019; Initial review: September 19, 2019; Accepted: May 07, 2020.

Objectives: To estimate the regional cutoff of optical density (OD) values for immunoglobulin M (IgM) antibodies against *Orientia tsutsugamushi* in serum and cerebrospinal fluid (CSF) for clinical diagnosis of scrub typhus and immunoglobulin G (IgG) antibodies in serum for sero-epidemiology in Gorakhpur, Uttar Pradesh, India. **Methods**: We used data from a serological investigation of acute encephalitis syndrome patients (*n*=407) during the 2016 outbreak in Gorakhpur, India to determine the cutoff for OD values for IgM antibodies, and from community-based serosurveys (*n*=1991) to estimate the cutoff for OD values for IgG antibodies. **Results**: We determined regionally relevant cutoff for OD values of 0.76 for IgM antibodies in serum and 0.22 in cerebrospinal fluid for scrub typhus diagnosis. For serosurveys, IgG antibody cutoff was 1.5. **Conclusions**: We have proposed locally relevant cutoffs for scrub typhus endemic regions, which may be useful for correctly classifying infected population.

Keywords: *Acute encephalitis syndrome, Diagnosis, Epidemiology, Immunoassay.*

Published online: **September 07, 2020; *PII*: S097475591600242**

Scrub typhus, caused by *Orientia tsutsugamushi* (OT), is the most common re-emerging rickettsial infection in India and many other South East Asian countries [1]. Although, various labo-ratory tests are available for diagnosis of rickettsial infection, Enzyme-linked immunosorbent assay (ELISA) based tests, particularly immunoglobulin M (IgM) capture assays can be made available at secondary and tertiary levels of healthcare [2]. The IgM ELISA manufactured by InBios (Scrub Typhus Detect, InBios International Inc., Seattle, USA) is considered as an alternative to the gold standard immunofluorescent assay (IFA) for diagnosis of acute infection [3].

IgM ELISA is meant for diagnostic purposes only, whereas IgG antibodies indicate recent and/or past exposure. IgG seroprevalence surveys are conducted to measure endemicity of OT infection in an area. In India, InBios IgM ELISA and IgG ELISA are commonly used for diagnosing scrub typhus as well as measuring endemicity of infection [4-10]. The manufacturer's instructions recommend calculation of regional cutoff for optical density (OD) values based on geographically representative serum samples [11,12]. Moreover, the IgM assay is recommended for diagnostic purposes using serum [11]; however, its applicability in cerebrospinal fluid (CSF) is not known. The present study was conducted to estimate the regional cutoff of OD values for IgM antibodies against OT in serum and CSF specimens for clinical diagnosis, and IgG antibodies in serum for sero-epidemiology.

METHODS

For this study, we used data collected during our previous two studies [5,13] in Gorakhpur, Uttar Pradesh, India, a highly endemic area for scrub typhus. The first was on 407 inpatients (aged ≤14 years) with a clinical diagnosis of acute encephalitis syndrome [14] during August to October, 2016. Blood and CSF samples were available for serological investigations for 389 and 374 patients, respectively. Sera and CSF were tested for IgM antibodies against OT using Scrub Typhus Detect ELISA. CSF was diluted in 1:10 proportion for detection of IgM antibodies [5]. The other was data from two community-based serosurveys conducted in Gorakhpur district to estimate prevalence of OT infection [13]. These surveys were conducted among healthy individuals in two

separate groups of villages in Gorakhpur district. Blood samples from 1991 individuals aged between 6 and 45 years were collected in these serosurveys, including 1085 during the phase-1 serosurvey, and 906 during the phase-2. Sera were tested for IgG antibodies against OT using Scrub Typhus Detect ELISAs following manu-facturer's instructions.

Statistical analyses: We used the following methods for deciding the cutoff for OD values of IgM and IgG antibodies against OT: (*a*) To determine the cutoff for OD values of IgM antibodies against OT in serum, we plotted the frequency distribution of OD values. The OD value corresponding to the anti-mode was considered as the cutoff. This method is often used for differentiating distribution of infected and un-infected individuals in tuberculosis infection surveys [15]. (*b*) We considered 356 AES patients for whom both serum and CSF samples were available for analyses [5]. We estimated the regression equation between OD values of IgM antibodies against OT in serum and CSF. Using this equation, we calculated the cutoff for OD value for CSF corresponding to the cutoff for serum OD. (*c*) We plotted the frequency distribution of OD values from healthy individuals enrolled in phase 1 and 2 of the serosurveys [13]. There was a bimodal distribution, with segregation of values at the two ends and a central portion of OD values close to the baseline. We considered OD value corresponding to anti-mode of distribution in phase-2 serosurvey and OD value corresponding to the beginning of distribution of infected individuals in phase-1 survey as cutoffs.

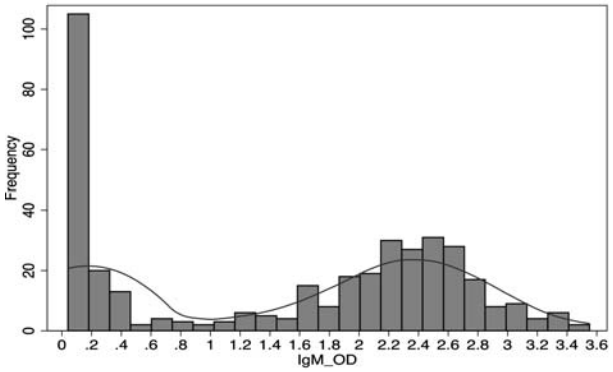


Fig. 1 *Frequency distribution of OD values for IgM antibodies against OT among 356 AES patients, Gorakhpur, Uttar Pradesh, 2016.*

RESULTS

The frequency polygon of OD values of IgM antibodies against OT in 389 AES patients showed a bimodal distribution, with anti-mode at 0.76. This was considered as cutoff for OD values against OT in serum IgM (***Fig.* 1**). A scatter diagram for the paired observations for OD values of IgM antibodies in serum and CSF showed a very strong positive correlation with a correlation coefficient of 0.83 (95% CI 0.79-0.86) (***Fig.* 2**). On linear regression analysis, the relationship between the OD values of IgM antibodies in serum and CSF was serum OD (Serum)=(1.07*CSF OD)+0.52. Based on this equation, for OD value of 0.76 for IgM antibodies in serum, the corres-ponding OD value for IgM antibodies in CSF was 0.224.

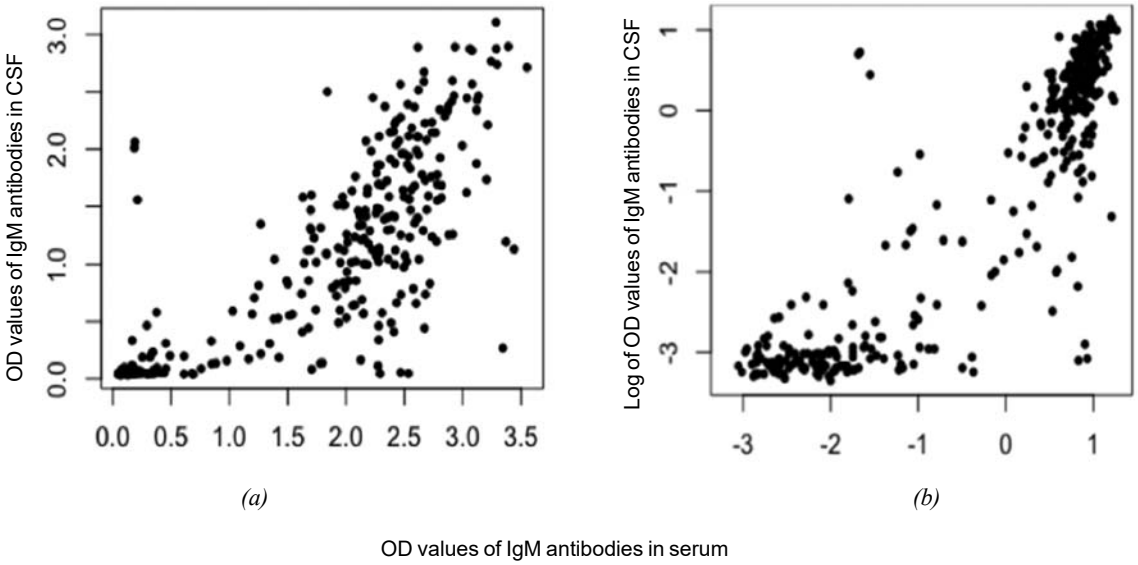
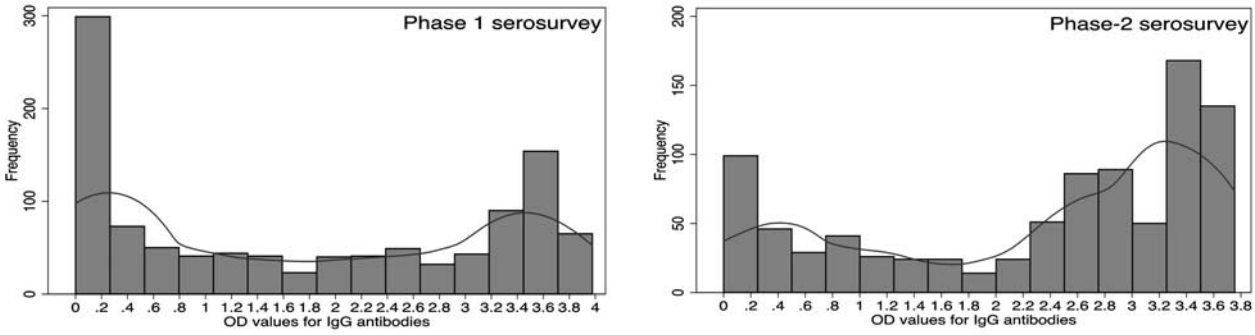


Fig. 2 *Scatter diagram showing (a) OD values and (b) log of OD values of IgM antibodies against OT in CSF and serum (n= 356).*



(a) *(b)*

Fig. 3 *Frequency distribution of OD values for IgG antibodies against OT in (a) phase-1 and (b) phase-2 surveys, Gorakhpur, Uttar Pradesh, 2016.*

The distribution of OD values from phase-2 survey showed an anti-mode at 1.5; however, the distribution from phase-1 survey did not reveal a clear demarcation between infected and uninfected individuals. The central portion between the two peaks was comparatively flat with OD values ranging from 0.6 to 2.5. OD value >2.5 in phase 1 corresponded to the distribution of infected individuals. In phase-1 and phase-2 serosurveys, 155 (14.3%) and 133 (14.7%) observations were between OD values of 1.5 and 2.5 (***Fig.* 3**).

DISCUSSION

Previous studies using Inbios ELISA kit have used OD value of 0.5 as the cutoff for IgM as well as IgG antibodies [4-10]. The manufacturer's instructions recommend calculation of cutoff value by determining the average of OD plus three times of the standard deviation (SD) of sera from healthy individuals and/or sera from persons with unrelated infections. It is further recommended that the end users calculate their cutoff using geographically relevant serum samples [11,12].

The phase-1 and phase-2 serosurveys were conducted among apparently healthy individuals at two different periods of transmission of scrub typhus infection in the community. However, using the OD values from children aged ≤14 years, the cutoff for IgM antibodies as per the kit recommended method, was 0.68 and 1.26 during the phase-1 and phase-2 surveys, respectively. The corresponding OD values for IgG anti-bodies was >3 in both the surveys. Higher cutoff obtained even during phase-1 survey, when the OT transmission in the community is expected to be low, indicates that the population included for sero-survey was not an unexposed population to OT. In view of this, we decided to consider the distributions of OD values for IgM among AES patients and IgG among healthy children for finding out the optimal cutoff.

The cutoff for IgM antibodies determined by us is higher than the cutoff of 0.5 observed by Blacksell, *et al*. [3] but comparable to the cutoff of >0.8 identified in another endemic area in India [18]. For IgG antibodies, A cutoff OD value of ≥1.5 in phase-1 serosurvey would have misclassified 14.3% individuals as infected, while a cutoff OD value of ≥2.5 in phase-2 serosurvey would have misclassified 14.7% infected individuals as uninfected. Since the primary objective of seroepidemiological studies is to estimate the disease burden, certain amount of misclassification in unavoidable with either cutoffs. The amount of misclassification; however, was not different with either cutoff. We therefore suggest an OD value of ≥1.5 as cutoff for classifying individuals as infected with OT for sero-epidemiological studies in Gorakhpur. With this cutoff, it was still possible to see clear transition for OT infections from 50.6% to 70.1% from phase-1 to phase-2 surveys. Trowbridge, *et al*. [16] have recommended a cutoff of >1.8 for IgG antibodies based on the community-based survey conducted in another high endemic setting in India.

Although the kit is recommended for detecting IgM antibodies only in serum samples, we observed good correlation between OD values for IgM antibodies against scrub typhus in serum and CSF. In comparison to serum where dilution of 1:100 is used, for CSF we used dilution of 1:10, as previously reported [17]. Since IgM antibodies cannot cross the blood brain barrier, the presence of such antibodies in CSF indicates that these antibodies are produced by antibody secreting cells in the central nervous system and hence presence of IgM antibodies against OT is more specific of scrub typhus infection. The calculated cutoff for CSF would require further evaluation before being used as a diagnostic criterion.

Our study has certain limitations. We did not use any gold standard test to compare the performance of Inbios

ELISA to calculate the cutoff for IgM and IgG antibodies. It was also not possible to calculate the cutoff based on manufacturer recommended procedure of mean (3 SD) based on endemic normal indivi-duals, in view of high endemicity of infection in the area.

In conclusion, we have calculated regionally relevant cutoffs for OD values of IgM in serum and CSF for clinical diagnosis, as well as cutoff for OD values for IgG antibodies for sero-epidemiological surveys in areas where OT transmission is endemic. Further evaluation of these methods may be used to find out accurate cutoffs in endemic areas, to correctly classifying infected population.

Acknowledgement: Dr Rashmi Arora, Former Head, Epidemiology and Communicable Diseases, Indian Council of Medical Research, New Delhi for her support and technical inputs on the study.

Contributors: MDG: conceived the study; MDG, SK, MM: designed the study protocol and were involved in sample/data collection; AM, SS, VB, JD: carried out laboratory investigations; MG, MDG: analysed the data; MDG, MG, DG, MM: inter-preted these data; MG, MDG: drafted the manuscript; DG, MM: critically revised the manuscript for intellectual content. All authors read and approved the final manuscript, and agree to be accountable for; all aspects of the manuscript.

Ethical clearance: The institutional ethics committee of National AIDS Research Institute, Pune; No. NARI EC/2015-24 dated 13 August, 2015 and NARI EC/2016-15 dated 12 September, 2015. National Institute of Epidemiology, Chennai; No.NIE/IHEC/201507/-01 and dated 20 July, 2016.

Funding: Indian Council of Medical Research, New Delhi; *Competing interest*: None stated.

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