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Application of Enzyme Linked Immunosorbent Assay (ELISA) and Indirect Fluorescent Antibody Test for Serodiagnosis of Acute Scrub Typhus in and Around Puducherry, India

Microbiology Section

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ABSTRACT

Introduction: Scrub Typhus (ST) is now endemic in India and is diagnosed by Immunochromatography based rapid kits or conventional ELISA to detect IgM antibodies. Clinical picture of ST may mimic other diseases like Dengue, Leptospirosis and viral haemorrhagic fevers. Indirect Fluorescent Antibody Test/ Immunofluorescence assay (IFA) is the reference standard which might help to confirm ST.

Aim: To critically analyse the practical utility of the gold standard IFA vis-à-vis ELISA in identifying acute ST in patients.

Materials and Methods: Archived acute blood samples from 140 febrile patients with clinical suspicion of acute ST, 70 patients with other febrile illnesses and 70 voluntary healthy blood donors were subjected to both ST IgM ELISA and ST IgM IFA.

Statistical Analysis: Chi-Square test was applied and p-values ≤0.05 were considered statistically significant.

Results: IgM IFA was positive in 120 out of 140 ST IgM ELISA positive ST patients and 16 out of 70 ST IgM ELISA negative febrile patients. All 70 blood donors were negative in both IgM ELISA and IgM IFA. Against the gold standard IFA, Sensitivity, Specificity, Positive Predictive value and Negative Predictive values for IgM ELISA were 88.24%, 86.11%, 85.71%, 88.57% respectively. Titres of ≥1:64 were considered as significant for IgM IFA.

Conclusion: It is recommended that standardisation is required regarding significant cut-off titre for IgM IFA in different regions of India. For serodiagnosis of acute ST, IgM ELISA still remains a viable alternative to IgM IFA in developing countries.

Keywords: Immunofluorescence assay, *Orientia tsutsugamushi*, Rickettsiosis, Zoonosis

INTRODUCTION

Scrub Typhus (ST) is caused by the Rickettsia *Orientia tsutsugamushi,* an intracellular gram negative bacilli which is transmitted by the bite of the infected Trombiculid mite (Chiggers). Now-a-days, ST has spread to areas apart from the 'Tsutsugamushi Triangle'. It is an important re-emerging zoonosis in India. The 'gold standard' serological test for the diagnosis of acute ST and other rickettsial infections is IgM Immunofluorescence Assay (IFA) [1]. However, immunochromatography/lateral flow assay based tests as well as ST IgM Enzyme Linked Immunosorbent Assay (ELISA) are commonly employed in many countries [2-6]. The non-specific Weil Felix test, in spite of its poor/moderate sensitivity, is still in use in resource poor countries.

Karp, Kato, Gilliam were the original prototypes of *Orientia tsutsugamushi* [1]. Other two serotypes Kawasaki and Boryong have been added later by Lee YM et al., [7]. Now, more than 20 serotypes have been reported [8]. ST IgM ELISA identifies antibodies against major recombinant outer membrane protein antigen [2] or a combination of recombinant protein from multiple strains of *Orientia tsutsugamushi* [3]. In IFA kits, only the most common four serotypes of *Orientia tsutsugamushi* are included viz., Karp, Kato, Gilliam and Boryong. This is the limitation of IFA. Since reports of ST diagnosis by IFA are few in India, this research was undertaken to diagnose acute ST by looking for IgM antibodies by ELISA as well as IFA. A critical analysis is made regarding the relevance of Scrub Typhus Inclusion Criteria (STIC) [9] and its modification [10] with particular reference to ST IgM IFA cut-off titre in Indian context.

MATERIALS AND METHODS

This is a prospective observational study based on diagnostic accuracy of tests and utilised archived and anonymised serum samples. This work was carried out in the Department of Microbiology, Mahatma Gandhi Medical College and Research Institute (MGMC and RI) Puducherry, during the period January 2017 to May 2018. Patients' selection was made on the basis of the following inclusion and exclusion criteria:

Inclusion criteria: Presence of typical eschar/fever with or without rash, chills and rigor/hepatosplenomegaly/lymphadenopathy/low platelet count/increased liver enzymes/fever with capillary leak syndrome.

Exclusion criteria: Immunocompromised status/bleeding disorders/Pulmonary tuberculosis etc., [11].

Additionally 70 healthy voluntary blood donors were included for control purpose. Our Institutional Human Ethics Committee (IHEC) approved this research project and granted waiver to retrieve the archived samples and proceed after anonymising the samples by way of excluding patients' identity (Project No: FACULTY/2015/04 dated 5.06.2015).

ST IgM ELISA

ST IgM ELISA plates were coated with 10 recombinant antigens of *Orientia tsutsugamushi*, targeting antibodies to the 56-kDa antigen. The kit used was ST Detect IgM ELISA (InBios International, Seattle, USA). Briefly, serum samples were first absorbed with Rheumatoid Factor (RF) sorbent to avoid false positivity and diluted 1:100 with the sample diluent and added to the micro-wells. The plates were incubated, washed, conjugate added and incubated further. Finally, substrate solution was added and the reaction was stopped by adding stop solution and the absorbance was read using an ELISA reader with a wavelength of 450 nm. Test was performed in strict adherence to the procedure outlined in the technical brochure and as described earlier [11,12]. Samples with Optical Density (OD)

values of ≥0.5 were taken as positive [13]. OD values of 140 ST IgM ELISA positive samples are listed in [Table/Fig-1].

Sl. No.	Range of OD Values in ST IgM ELISA	No. of Positive Samples
1	≥0.502-0.700	8
2	≥0.701-0.900	6
3	≥0.901-1.100	34
4	≥1.101-1.300	43
5	≥1.301-1.500	2
6	≥1.501-1.700	5
7	≥1.701-1.900	2
8	≥1.901-2.100	5
9	≥2.101-2.300	2
10	≥2.301-2.500	3
11	≥2.501-2.700	6
12	≥2.701-2.900	2
13	≥2.901-3.000	1
14	≥3.001-3.976	21
Total		140

[Table/Fig-1]: OD values for ST IgM ELISA Positive samples (n=140).

ST IgM IFA

ST IFA Kits from Fuller Laboratories (OTM-120 Fuller Laboratories, Fullerton, California, USA) were used. IFA slides were coated with four different prototype antigens of *O. tsutsugamushi* namely Karp, Kato, Gilliam and Boryong. IFA was performed strictly adhering to the kits' protocol. Briefly, Patients' sera were diluted 1:64 in IgM serum diluent, added to the IFA slides and incubated for 30 minutes to allow reaction of serum antibody with four *Orientia tsutsugamushi* serotypes. Then conjugate was added to label the antigen-antibody complexes, and incubated for 30 minutes. The slides were washed to remove non-reactive serum proteins, washed again to remove non-reactive conjugate, dried and mounted with the mounting medium and read with ×400 magnification, at 390 nm using Primo Star iLED Fluorescent microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Positive reactions may then be retested at higher dilutions to determine the highest end point dilution. As per the kit, the cut-off titre of ≥1:64 for IFA IgM were considered positive. Positivity in any one/or more of the four serotypes was taken as IFA positivity.

STATISTICAL ANALYSIS

Based on the 85% sensitivity of the antibody tests, sample size (n) was calculated using the formula:

$$n = \frac{1.96^2 (PQ)}{d^2}$$

P=0.85 (Sensitivity of the antibody tests)

Q=1-p=0.15

d^2=0.06 (Margin of error at 6%).

The sample size was therefore 136 and it was rounded upto 140.

The sample included 140 antibody positive patients in ST IgM ELISA, 70 antibody negative febrile patients with causes other than ST. Additionally 70 healthy voluntary blood donors were included. Thus a total of 280 serum samples were investigated in this study. Sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) between ELISA and IFA were calculated using Graph Pad Quick Calcs (Graph Pad Software Inc, USA) and p-value ≤0.05, was considered as statistically significant. For other parameters, Chi-square and Kappa statistical analysis was performed using IBM SPSS Statistics 17 for Windows (SPSS Inc; Chicago, USA).

RESULTS

Out of 210 febrile patients, 136 were positive for IgM IFA (120 among 140 IgM ELISA positive patients and 16 from 70 ELISA negative patients). Altogether a total of 156 cases were considered as ST patients viz., 120 positive in both ST IgM ELISA and ST IgM IFA, 20 positive only in IgM ELISA and 16 positive only in IgM IFA. All 70 healthy blood donors were negative for IgM antibodies in ELISA as well as IFA. [Table/Fig-2] compares results of ST IgM ELISA vs ST IgM IFA with reference to positivity for Kato/Karp/Boryong/Gilliam serotypes in various combinations. Results of statistical analysis of ELISA vs IFA for IgM is shown in [Table/Fig-3]. IgM ELISA has shown a sensitivity of 88.24% and specificity of 86.11%. Clinical details and laboratory results of 156 patients confirmed as ST by IgM ELSA and/or IgM IFA are presented in [Table/Fig-4], which compares the presentation in children vs adults. Typical ST eschar was observed on 30 patients only (19.23%). There are few parameters such as abdominal pain and malaise that were seen in more number of adults than children (p≤0.05). However, higher number of children had elevated liver enzymes than adults (p≤0.05). Acute Respiratory Distress syndrome (ARDS) and other complications among these 156 cases of scrub typhus are mentioned in [Table/Fig-5].

Sl. No.	Combination of ST IgM IFA	ST IgM ELISA Positive (n=140)	ST IgM ELISA negative (n=140)	Total (n=280) (%)
1	Kato+Karp+Boryong+Gilliam	60	4	64 (22.85)
2	Karp+Kato+Boryong	2	1	3 (1.07%)
3	Karp+Boryong+Gilliam	4	-	4 (1.42%)
4	Boryong+Gilliam+Kato	7	1	8 (2.85%)
5	Gilliam+Kato+Karp	5	-	5 (1.78%)
6	Kato+Gilliam	7	1	8 (2.85%)
7	Karp+Gilliam	3	1	4 (1.42%)
8	Boryong+Gilliam	14	2	16 (5.71%)
9	Kato only	1	-	1 (0.5%)
10	Boryong only	2	-	2 (0.71%)
11	Gilliam only	15	6	21 (7.5%)
12	**Total positive**	**120**	**16**	**136 (48.57%)**
13	All Negative	20	124	144 (51.42%)
	Total	**140**	**140**	**280**

[Table/Fig-2]: Comparison of IgM ELISA with various combinations of IFA (n=280).
ST IgM IFA Positivity of different serotypes (n=136):
Karp → 80; Kato → 89; Boryong → 97; Gilliam → 130

Tests	Kappa (95% Confidence Interval)	Sensitivity (95% Confidence Interval)	Specificity (95% Confidence Interval)	Positive Predictive Value (95% Confidence Interval)	Negative Predictive Value (95% Confidence Interval)
ST IgM ELISA Vs ST IgM IFA	0.743 (0.664 to 0.821)	88.24% (81.60 to 93.12)	86.11% (79.37 to 91.31)	85.71% (79.91 to 90.05)	88.57% (82.96 to 92.50)

[Table/Fig-3]: Sensitivity and Specificity of ST IgM ELISA Vs. ST IgM IFA (n=280).

Serological response in 156 patients from whom the samples were collected in the first week and second week of febrile illness is presented in [Table/Fig-6]. Higher positivity of 64.10% was observed in samples collected between eight to 15 days, compared to 35.90% positivity in the samples collected during the first week of fever (3 to 7 days). However, the difference is not statistically significant (p=0.1760). Positive reaction was visualised as small sharply defined green fluorescent rods within each antigen spot. Counterstained (red) cells alone seen in negative reaction. Images of IFA positive and negative samples are shown in [Table/Fig-7].

Clinical/Laboratory findings	Children (0-18 years) (n=52)	Adults (≥19 years) (n=104)	Total (n=156) (%)	p-values* (Chi-square/ Fisher's exact test)
Fever 3 to 7 days	23	48	71 (45.51)	0.8201
Fever 8 to 15 days	29	56	85 (54.48)	0.8201
Chills and Rigor	22	52	74 (47.43)	0.3644
Malaise	11	44	55 (35.25)	**0.0091**
Headache	16	49	65 (41.66)	0.5092
Myalgia	19	40	59 (37.82)	0.8154
Abdominal Pain	24	72	96 (61.53)	**0.0052**
Nausea	12	38	50 (32.05)	0.0894
Vomiting	17	41	58 (37.17)	0.4122
Cough and Expectoration	26	48	74 (47.43)	0.6502
Pneumonitis	5	8	13 (8.33)	0.7610
Eschar	13	17	30 (19.23)	0.1961
Lymphadenopathy	8	15	23 (14.74)	0.8731
Hepatomegaly	9	14	23 (14.74)	0.5229
Splenomegaly	3	9	12 (7.69)	0.7519
Rash	2	3	5 (3.20)	0.7479
Pedal oedema	0	5	5 (3.20)	0.1700
Elevated Liver Enzymes (AST/ALT/ALP)†	9	7	16 (10.25)	**0.0401**
Creatinine (>1.0 mg/dL)	7	18	25 (16.02)	0.4244
Thrombocytopaenia (<1.5 lacs/mm^3)	11	19	30 (19.23)	0.6665
Leucocytosis (>11,000 mm^3)	8	15	23 (14.74)	0.8731

[Table/Fig-4]: Clinical findings and laboratory results of acute Scrub Typhus cases: Comparison between children and adults (n=156).
*p-values <0.05 were considered significant; †AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline phosphatase

Clinical/Laboratory findings	Children (0-18 years) (n=52)	Adults (≥19 years) (n=104)	Total (n=156) (%)	p-values* (Chi-square/Fisher's exact test)
ARDS	2	5	7	0.7845
Acute renal failure	0	1	1	0.4781
Myocarditis	0	3	3	0.5363
Hepatic dysfunction	0	1	1	0.4781
Septic shock	0	2	2	0.8013

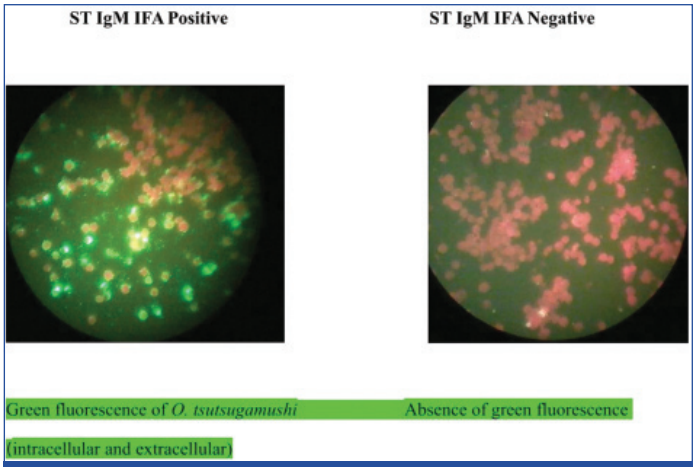
[Table/Fig-5]: Complications of scrub typhus.

Category	Fever 3 to 7 days (n=81)	Fever 8 to 16 days (n=129)	Total
Concordance (patients Positive for ELISA and IFA)	45	75	120
Concordance (patients Negative for ELISA and IFA)	25	29	54
IFA Positive, but ELISA Negative	5	11	16
ELISA Positive, but IFA Negative	6	14	20
Total	81	129	210

[Table/Fig-6]: Comparison of results of ST IgM ELISA vs ST IgM IFA (n=210) with reference to duration of fever.

DISCUSSION

ST has become an endemic disease in Puducherry and surrounding Tamil Nadu, necessitating routine testing for ST in all cases of acute febrile illnesses. ST IgM IFA kits of Fuller Laboratories, USA has been validated and found to be satisfactory by overseas and Indian researchers [14-17]. This is also the experience with this kit, on the basis of this research. Unlike ELISA, IFA is a quantitative test, subjective in nature, demand good technical experience and expensive. ELISA is a qualitative test, but it is objective, does not require high technical expertise and is cost effective. The major drawback with the gold standard IFA is the prevalence of diverse *O.tsutsugamushi* serotypes in different parts of the world. The complete profile of *O.tsutsugamushi* genotypes circulating in India is yet to be compiled. This might lead to a poor sensitivity of IFA inspite of its remarkable specificity.



[Table/Fig-7]: ST IgM positive and negative cases.

Sensitivity of IFA varies from 42% to 100% according to different authors. Thus, Kim DM et al., observed a sensitivity of 42% in blood samples collected before seven days of fever, and steadily increasing to 76%, 84%, 96% in second, third and fourth weeks of illness [18]. Blacksell SD et al., reported 69% and 86.20% in admission and paired samples respectively [19]. La Scola B et al., demonstrated a sensitivity of 84%, 70%, 48% and 54% with IgM IFA cut-off titres of ≥1:100, ≥1:200, ≥1:400 and ≥1:1200 respectively [1]. Lim C et al., Coleman RE et al., and Stephen S et al., reported sensitivity of 81.5%, 85% and 75% respectively [10,20,21]. Recently Pote K et al., observed that at a cut-off titre of ≥1:64, Fuller labs ST IgM IFA kit has a sensitivity of 96.8% [16]. Phetsouvanh R et al., noted a sensitivity of 95% [22]. Gupta N et al., and Wongchotigul V et al., reported a sensitivity of 100% at a cut-off titre of 1:64 and ≥1:400 respectively [15,23].

Performance of ST IgM InBios ELISA kit was found to be equal to IFA IgM Kit [24] or some times better than the gold standard IFA [16]. In the present study, against the gold standard IFA, ELISA has a Kappa factor of 0.743, which is considered to be 'good'. Cut-off titres for ELISA and IFA must be considered for different geographical regions. ST IgM ELISA InBios kit's cut-off OD values for India has been set at 0.5 [13], while some authors consider 0.8 [25] and 1.0 [17]. Regarding IgM IFA test, the cut-off titres of ≥1:64 for IFA IgM was considered as per the recommendation of the kit. Positivity in any one/or more of the four serotypes Kato, Karp, Boryong and Gilliam was taken. The seropositivity of ST patients to more than one serotype indicates antigenic cross reactivity among them. For serological diagnosis of acute ST, presence of IgM antibody is considered, since IgG positivity might point to past infection/secondary infection. Significant ST IgM IFA titre of ≥1:64 were considered by Pote K et al., recently from Central India [16]. As per STIC, serological confirmation of ST can be made by any one of the following two conditions [9]:

1. IgM IFA titre in acute samples should be ≥1:10,240
   or
2. Seroconversion/four-fold increase in IgM IFA titres in paired sera (≥1:10,240).

To demonstrate seroconversion/four-fold increase in titre of IFA, convalescent samples from patients is required. However, patients coming from rural and remote areas may find it difficult to come again for the second visit. IgM ELISA is not included in

STIC, for being a qualitative test. This is inspite of the fact that the ELISA is still considered as a viable alternative to the highly subjective, technically demanding and expensive IFA, because of its simplicity, affordability and availability in India [11-13,15-17,19]. STIC is yet to get universal approval. Modified STIC by Lim C et al., has substantially lowered the IgM IFA titre from ≥1:10,240 to ≥1:3,200 [10]. Even this titre is not reached by Indian patients as reported by many Indian rickettsiologists and their cut-off titres ranged from 1:64 [15,26], ≥1:128 [14,17] to ≥1:256 [27]. Kim DM et al., from Korea and Tantibhedhyangkul W et al., from Thailand considered IgM/IgG IFA cut-off titres of ≥1:400 or a fourfold increase in convalescent samples as significant [18,28]. According to Sonthayanon P et al., a single IFA IgM titer of ≥1:400 or a 4-fold or greater rise in IFA IgM titre points to ST [29]. Therefore, there is an urgent need to address this issue and define the optimal cut-off titre in countries like India so as to understand the true prevalence of ST in different geographical regions. Incorporation of local strains of *O. tsutsugamushi* in the serological kits would help in identifying more cases of ST.

There are instances of false positivity with ST IgM ELISA due to other febrile diseases like Typhoid, Malaria, Leptospirosis, Dengue and Pulmonary tuberculosis [30,31]. The earlier understanding was that in ST patients IgM antibody appears early, stays at significant level only for about a month whereas IgG appears late during second or third week of febrile illness and persists for several months [31]. However, recent report by Varghese GM et al., changed this concept as they have demonstrated the persistence of IgM and IgG antibodies in ST patients' upto 13 and 36 months respectively [25]. Hence in endemic areas, ST IgM positivity has to be taken with caution and requires clinical correlation. Molecular tests aimed at detecting *O.tsutsugamushi* DNA would be helpful in confirming present infection.

LIMITATION AND FUTURE RECOMMENDATION

A total of 280 participants were tested. Larger number of cases and controls might perhaps give a clear picture of ST prevalence in this region. ST IgM IFA kits need to be made available at affordable cost and efforts should be made to manufacture them in India, incorporating local isolates of *O. tsutsugamushi.*

CONCLUSION

ST has to be included in the list of acute febrile illnesses in India. ST IgM IFA kit needs further evaluation throughout India so as to decide the optimal cut-off titres in different geographical locations. Until this is achieved, perhaps the best alternative and only viable option available for India is to continue with ST IgM ELISA using appropriate cut-off OD values adjusted to the respective geographical regions.

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