Journal of Applied Biology & Biotechnology Vol. 8(04), pp. 65-68, July-Aug, 2020
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2020.80410

Comparison of diagnostic performance between serological tests (Scrub IgM-ELISA) and Scrub-PCR for scrub typhus fever in an Indian tertiary care teaching hospital

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ARTICLE INFO

Article history:
Received on: March 17, 2020
Accepted on: April 30, 2020
Available online: July 30, 2020

Key words:
Scrub typhus, PCR, IgM, *Orientia tsutsugamushi,* serology, diagnostic.

ABSTRACT

Scrub typhus is a mite borne transmittable disease caused by the obligate intracellular bacterium known as *Orientia tsutsugamushi*. Although it presents with nonspecific clinical manifestation, early diagnosis and treatment often prevents it's complication. Here in this study, we used two different diagnostic tools Scrub-polymerase chain reaction (PCR) and serologic Scrub IgM antibody based enzyme-linked immunosorbent assay (IgM ELISA) test. Out of 154 cases, total of 101 cases were found Scrub IgM-ELISA positive. Both serology and Scrub-PCR was done in total 96 cases. Out of 96 cases, 41.6% (*n*–40) were Scrub-PCR positive and 58.3% (*n*–56) cases were PCR negative. Eighty percent cases become serology positive (IgM ELISA) those who presented after 7 days but before 14 days of illness and least (54%) when presented before 1 week of illness. Twenty cases were found to be IgM positive but PCR negative. The mean duration of illness among those 20 patients who were IgM positive but PCR negative was found to 12.55 days. Only four cases were PCR positive but IgM negative. The mean duration of illness was 6.2 days among those four patients so that the antibody conversion was not detectable at that phase. Therefore, the combination of molecular and serological tests needs to be used, so that it can cover the entire duration of illness for early and accurate diagnosis so as to prevent the complications.

1. INTRODUCTION

Scrub typhus, also called as Tsutsugamushi disease, is highly endemic in region called tsutsugamushi triangle. Globally, it is estimated that people at risk of scrub typhus is approximately one billion and average incidence of cases on worldwide is one million [1]. The most common presenting symptom is acute fever and it often occurs with other symptoms, such as cough, nausea, breathlessness, vomiting, rash, headache, and myalgia. The severity of infection may be varying from milder form to a fatal outcome, and mortality approximately ranges from 35% to 50%, with multi-organ dysfunction [2]. Because of high variability and lack of specificity in clinical presentation, it is difficult to diagnose clinically this infection. Mild transaminitis is a very consistent laboratory finding in Scrub typhus [3]. The sensitivity and specificity of Weil–Felix test in comparison to other diagnostic tests are less. For diagnosis, current serological test of choice is IgM ELISA. Gold standard is immunofluorescence assay (IFA). The *polymerase chain reaction* (PCR) techniques have played a major role in diagnosis of infectious and non-infectious diseases [4,5]. For the diagnosis of tsutsugamushi disease, the use of PCR techniques, like conventional PCR [6], Nested PCR (N-PCR) [7–9] and Real time PCR [10] have a good description in various studies. A handful of studies over the years have given an insight toward geographical distribution and epidemiology of scrub typhus from few parts of India. However, considerably more research is still required to clarify the full picture across India. Furthermore, the performance of the newer assays, such as IgM antibody based enzyme-linked immunosorbent assay (IgM ELISA) and N-PCR, in statistically significant Indian population was lacking.

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The present study was undertaken to fill the research gaps related to the performance of serological and molecular tests in diagnosis of scrub typhus in a tertiary care teaching hospital.

2. MATERIALS AND METHODS

This prospective observational study was carried out in department of paediatrics of IMS and SUM hospital among the hospitalized children from age 1 month to 14 years with approval of institutional ethical committee. Those who were admitted with history of acute onset febrile illness(undifferentiated) of more than 5 days without any cause, with one or more clinical manifestations, such as pain in abdomen, cough, headache, rash, lymphadenopathy, organomegaly, oedema, and eschar were enrolled in the study. Other common clinical conditions which mimic scrub typhus, such as malaria, dengue, enteric fever, and leptospira, were excluded by doing specific laboratory test. All the patients admitted as suspected scrub typhus with above-mentioned clinical features underwent serological test (Scrub IgM-ELISA), and both serology (Scrub IgM-ELISA) and PCR was done in total 96 cases only.

IgM ELISA assay is an ELISA test kit for the identification of IgM antibodies to *Orientia tsutsugamushi* bacteria in the serum of human beings. Scrub Typhus Detect™ IgM ELISA (In Bios International Inc, Seattle, WA) kit was used to detect the presence of IgM antibodies in the suspected patient's serum. Recombinant 56 kDa type specific antigen of *O. tsutsugamushi*, Karp, Kato and Gilliam genotypes were coated in the wells of ELISA. According to the manufacturer's instruction, suspected patients' blood (sera) at a dilution of 1:100 was assayed. Absorbance was read at 450 nm. The cut off optical density (OD) value was fixed at 0.5, which was determined experimentally following kit protocol.

The Genomic DNA was isolated from blood using Hipura DNA blood mini kit (Himedia, India). It needs 200 µl fresh bloods for isolating the Genomic DNA according to the protocol available in the kit. 25 µg/µl intact DNA was used for PCR amplification. The nested-PCR assay was performed as described by Furuya *et al.* [4] with slight modification. The oligonucleotide primers used were based on the nucleotide sequences of a gene encoding for the 56 kDa antigen of a Gilliam strain of *O. Tsutsugamushi*. Primers F1 (5′-TCA AGC TTA TTG CTA GTG CAA TGT CTGC-3′) and R1 (5′-AGG GAT CCC TGC TGC TGT GCT TGC TGC G-3′) were used to amplify a 1,003 bp fragment, then nested primers F2 (5′-GAT CAA GCT TCC TCA GCC TAC TAT AAT GCC-3′) and R2 (5′-CTA GGG ATC CCG ACA GAT GCA CTA TTA GGC-3′) were used to amplify a 483 bp fragment. The PCR products were electrophorized in 1.5% agarose gel (Boehringer Mannheim, Germany). After electrophoresis, the agarose gel containing DNA fragments was stained in 0.5 g/ml of Ethidium bromide and visualized by ultraviolet transilluminator.

3. RESULTS

Totally 154 clinically suspected probable scrub typhus cases were enrolled in the 2-year study period. Scrub IgM ELISA was done in total 154 cases but Scrub-PCR was done only in 96 patients. Out of 154 patients, 105 cases were confirmed to be positive for scrub typhus:- 101 tested positive for Scrub IgM-ELISA and four tested positive for scrub PCR alone (negative for IgM ELISA). Out of 105 confirmed scrub typhus cases, majority of the patients (50.5%) were in age group of 1–5 years followed by 26.73% in the age group of 5–10 years, 11.4% in age group of less than 1 year and in the age group of 10–14 years. Mean age of hospitalization was 5.63 years and the youngest age of the child was 6 months. Male and Female ratio was 1.69. The laboratory findings observed in our study were Leucocytosis (53.3%), thrombocytopenia (42%), and deranged liver function test (10.4%).

3.1. Distribution of Patients According to Scrub IgM-ELISA Titer (*n*−154)

Out of 154 cases, total 101 cases were Scrub IgM-ELISA positive. In majority of patients (43%) observed scrub IgM titer

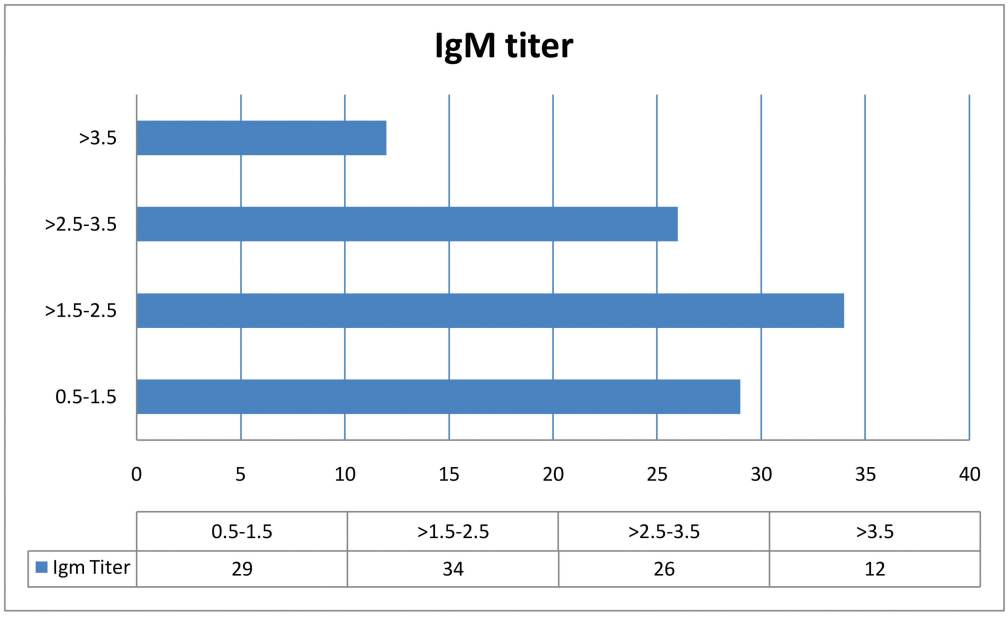

Figure 1: Distribution of patients according to Scrub IgM ELISA Titer.

was between 1 and 2 OD and least 10% of cases observed titer was between 0.5 and 1 OD (Figure 1).

3.2. Distribution of Cases Positive for Each Scrub Typhus Test Performed

Out of 154 samples sent for IgM ELISA, 101 cases (65.5%) were positive. Both serology and Scrub-PCR was done in total 96 cases. Out of 96 cases, 41.6% (*n*−40) Scrub-PCR positive, (IgM ELISA-positive) and 58.3 (*n*−56) cases PCR negative (Table 1).

3.4. Scrub IgM-ELISA Positivity According to the Duration of Illness

Out 154 samples tested by Scrub IgM-ELISA, 101 samples (65%) were positive. Majority (87.5%) of samples were positive when patients presented between the 10 and 14 days of illness, followed by 78.9% when patients presented between 7 and 10 days of illness. Those patients who presented after 2 weeks of illness only 60% of patients IgM were positive. In 54% of cases, IgM ELISA were positive when presented within the 5–7 days of illness (Table 2).

3.5. Scrub-PCR Positivity According to the Duration of Illness

Out of 96 samples tested by Nested PCR, 40 samples were PCR positive. Majority of patients (45.8%) were PCR positive when presented to hospital within 7 days of illness, followed by 44.4% when presented 7–10 days of illness. In 20% cases, PCR came positive when patients presented to hospital between 10 and 14 days of illness. Only a single sample out of seven tested (14.2%) was positive for scrub antigen also after the 2 weeks of illness (Table 3).

Table 1: Distribution of cases positive for each scrub typhus test performed.

Scrub IgM (154)	Positive = 101
	Negative = 53
Scrub PCR(96)	Positive = 40
	Negative = 56

Table 2: Scrub IgM ELISA positivity according to the duration of illness.

Duration of illness	No of sample tested	Positive	Percentage (%)
5–7 days	74	40	54
7–10 days	57	45	78.9
10–14 days	8	7	87.5
>14 days	15	9	60
Total	154	101	65

Table 3: Scrub PCR positivity according to the duration of illness (*n* = 96).

Duration of illness	No. sample sent	Positive	Percentage (%)
5–7 days	48	22	45.8
7–10 days	36	16	44.4
10–14 days	5	1	20
>14 days	7	1	14.2
Total	96	40	41.6

3.6. Co Relations of IgM ELISA and Scrub PCR

Totally 154 clinically suspected scrub typhus samples collected over 2-year study period. Out of 154 samples only 96 samples were sent for both serology and N-PCR testing. Among those 96 samples, 40 were N-PCR positive. Twenty cases were found to be IgM positive but PCR negative. The mean duration of illness among those 20 patients (IgM positive but PCR negative) were found to 12.55 days so that the antigen detection may be negative at that stage. Because of late hospitalization and previous administration of Azithromycin giving rise to low antigen level which could not be detected by nested PCR leading to low positivity rate. Only four samples had PCR positive but IgM negative. The mean duration of illness was 6.2 days among those four patients indicating that the antibody conversion was not detectable at that phase (Table 4).

4. DISCUSSION

The IgM ELISA kits for scrub typhus diagnosis have become the test of choice in recent years at many parts of the Indian subcontinent, for the fact that the test kits are available commercially. It also does not require dedicated instrumentation except for an ELISA OD reader and more importantly the ease in interpretation of assay. However, careful consideration must be given to determine the cut off OD prior to utility of IgM ELISA in routine diagnosis at any given endemic area. In our study, totally 96 patients were tested for both IgM ELISA and N-PCR. Out of 96 individuals, 56(57%) patients positive for IgM ELISA, 36(37.5 %) individuals positive for both IgM ELISA and N-PCR. In our study, we observed that majority of the cases were IgM ELISA positive compared to N-PCR, it was due to late hospital admission (mean 8.95 days) and administration anti-rickettsial antibiotics (Azithromycin) at the peripheral hospital. Because of late hospitalization and previous administration of azithromycin giving rise to low antigen level which could not be detected by nested PCR leading to low positivity rate. In our research study, we mostly compared IgM ELISA with Nested PCR for diagnosis of Scrub typhus through we did not apply the gold standard method IFA for the accuracy of diagnosis.

N-PCR is known to be 100 times more sensitive over a single PCR and we preferred using N-PCR as one of the molecular test in our study for diagnostic validation. The treatment prior to the collection of blood samples for scrub typhus has profound effect on the molecular test results. The blood samples collected from the suspected patient were used as clinical material to extract DNA for molecular diagnosis. Nested PCR was one of the very sensitive test and the detection method for the first primer pair was (F1 and R1) 25 ng per assay and for the second primer pair was (F2 and F2) 25 ng of DNA [11]. Kim *et al.* [12] observed that the sensitivity of N-PCR on buffy coats was 90.5% before initiation of antibiotics which decreased to 60.5% within 3 days of anti rickettsial antibiotics, further the sensitivity decreased to as

Table 4: Correlations of IgM ELISA and Scrub PCR (*n* = 96).

	N-PCR positive (40)	N-PCR negative (56)
IgM Positive(56)	36	20
IgM Negative(40)	4	36

less as 10% by the 4th day. Molecular tests can detect the genetic material of *O. tsutsugamushi* even before the febrile episode sets in. It is also observed that there is a sero negative period during the first week of illness [13], limiting the use of serological tests in this stage of illness. Considering only untreated cases, N-PCR was positive in 96.8% of the cases indicating the clinical usefulness of N-PCR in optimal selection of treatment. It was observed from our study that, there were four cases among the 96, which were only positive by N-PCR and negative for serology. These four cases had history of fever of less than 1 week (mean 6.25 days) indicating high antigenic load and the early sero negative stage in scrub typhus. In such early stage of the illness, N-PCR or other molecular test could be the only test of choice for diagnosis of scrub typhus. Other molecular targets in scrub typhus diagnosis evaluated were 150-, 110-, 72-, 58-, 56-, 49-, 47-, and 20-kDa genes by Oaks et al. [14,15] and was found that there are other targets of diagnostic importance in scrub typhus, however, require thorough validation. The diagnosis of scrub typhus is further complicated by non-availability of sensitive and specific diagnostic tests in many part of the country. Serological assays were the single most group of assays aiding in laboratory diagnosis of scrub typhus till now because of its wide availability, cost effectiveness and easy implementation. With the newer diagnostic tests available such as IFA and molecular techniques, such as nested PCR (N-PCR) and quantitative PCR (Q-PCR), the diagnosis of the disease can be made in a very early stage leading to decrease morbidity and mortality.

5. CONCLUSION

In last few years, there has been resurgence of scrub typhus cases in Indian sub-continent. Immunofluorescent assay is the current gold standard test in diagnosis of scrub typhus but serological assays were the single most group of assays aiding in laboratory diagnosis of scrub typhus till now. However, the challenges, such as requirement of homologous antigens, variable cut off titre across endemic countries, and subjectivity of the assays are some of the major drawbacks of this assay. Furthermore, serological assays are generally negative in the early stage of the illness; thereby limiting the utility of serological tests in this stage of the illness. Serological assays continue to be the most common tests in diagnosis of rickettsial diseases. Molecular test have become popular over the years as the detection of the disease become earlier than that of serological tests and are specific in single acute clinical specimen. Hence, a combination of molecular and serological tests needs to be used so that it can cover the entire duration of illness. Further work is needed to develop methods that aid in rapid diagnosis of scrub typhus such as tests that are based on antigen detection in blood or urine.

CONFLICT OF INTEREST

Authors declare that they do not have any conflicts of interest.

FINANCIAL SUPPORT

None.

REFERENCES

1. Watt G, Paro P. Scrub typhus and tropical Rickettsioses. Curr Opin Infect Dis 2003;16(5):429–36.
2. Kawamura A, Tanaka H. Rickettsiosis in Japan. Jpn J Exp Med 1988;58:24–8.
3. Mahajan SK, Rolain JM, Kashyap R, Bakshi D, Sharma V, Prasher BS, et al. Scrub typhus in Himalayas. Emerg Infect Dis 2006;12:1590–2.
4. Furuya Y, Yoshida Y, Katayama T, Yamamoto S, Kawamura A, Jr. Serotype-specific amplification of Rickettsia *tsutsugamushi* DNA by nested polymerase chain reaction. J Clin Microbiol 1993;31(6):1637–4.
5. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 1988;239(4839):487–91.
6. Sugita Y, Nagatani T, Okuda K, Yoshida Y, Nakajima H. Diagnosis of typhus infection with Rickettsia *tsutsugamushi* by polymerase chain reaction. J Med Microbiol 1992;37(5):357–60.
7. Saisongkorh W, Chenchittikul M, Silpapojakul K. Evaluation of nested PCR for the diagnosis of scrub typhus among patients with acute pyrexia of unknown origin. Trans R Soc Trop Med Hyg 2004;98(6):360–6.
8. Sharma A, Mahajan S, Gupta ML, Kanga A, Sharma V. Investigations of an outbreak of scrub typhus in the Himalayan Region of India. Jpn Infect Dis 2005;58:208–10.
9. Cui X, Shi Y, Zhao L, Gu S, Wei C, Yang Y, Wen S, Chen H, Ge J. Application of real-time quantitative PCR to detect mink circovirus in naturally and experimentally infected minks. Front microbiol 2018;9:937.
10. Stenos J, Graves SR, Unsworth NB. A highly sensitive and specific real-time PCR assay for the detection of spotted fever and typhus group Rickettsiae. Am J Trop Med Hyg 2005;73(6):1083–5.
11. Sarangi R, Pradhan S, ch Debata N, Mahapatra S. Clinical profile of scrub typhus in children treated in a tertiary care hospital in eastern India. Pediatria Polska 2016;91(4):308–11.
12. Saisongkorh W, Chenchittikul M, Silpapojakul K. Evaluation of nested PCR for the diagnosis of scrub typhus among patients with acute pyrexia of unknown origin. Trans R Soc Trop Med Hyg 2004;98(6):360–6.
13. Kim DM, Byun JN. Effects of antibiotic treatment on the results of nested PCRs for scrub typhus. J Clin Microbiol 2008;46(10):3465–6.
14. Burgdorfer W. Hemolymph test a technique for detection of rickertsiae in ticks. Am J Trop Med Hyg 1970;19(6, pt. 1):1010–4.
15. Oaks EV, Stover CK, Rice RM. Molecular cloning and expression of Rickettsia *tsutsugamushi* genes for two major protein antigens in Escherichia coli. Infect Immun 1987;55(5):1156–62.

How to cite this article:
Gatika A, Swain SK, Mohanty JN, Sarangi R. Comparison of diagnostic performance between serological tests (Scrub IgM-ELISA) and Scrub-PCR for scrub typhus fever in an Indian tertiary care teaching hospital. J Appl Biol Biotech 2020;8(04):065–068. DOI: 10.7324/JABB.2020.80410