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Research Article

POLYMERASE CHAIN REACTION FOR THE DIAGNOSIS OF SCRUB TYPHUS: A PROSPECTIVE STUDY

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A B S T R A C T

Background: Scrub typhus is a rickettsial infection which is caused by Orientia tsutsugamushi and transmitted by the bite of chigger. It is an important cause of acute undifferentiated febrile illness. Delay in diagnosis may prove to be life threatening.
Objective: The aim of this study was to determine the diagnostic accuracy and clinical usefulness of polymerase chain reaction (PCR) for the diagnosis of scrub typhus through a comparison of PCR and IgM Micro-IFA with IgM ELISA positive cases.
Material and Methods: All cases clinically suspected of scrub typhus over a period of 1 year were analysed. EDTA blood and sera were collected for PCR and serologic evaluation by IgM ELISA and IgM Micro- IFA respectively. **Results:** Out of 327 clinically suspected cases of scrub typhus, 177 cases were positive by IgM ELISA which were further subjected to IgM Micro- IFA and PCR. Out of 177 IgM ELISA positive cases, 174(98.3%) cases were positive for IgM IFA and 17(9.6%) cases were positive by PCR. The results of PCR showed a sensitivity and specificity of 9.6% and 100% respectively. **Conclusion:** Due to uncommon presentation of rash, lymphadenopathy, eschar heavy reliance on these features to clinically diagnose scrub typhus should be discouraged. PCR showed high specificity but low sensitivity as most patients had received antibiotics. Late presentation in most cases decreased chances of isolating DNA from the blood.

INTRODUCTION

Scrub typhus is a zoonotic disease caused by an obligate intracytoplasmic bacterium, *Orientia tsutsugamushi* and transmitted by the bite of chigger. It is a public health problem in Asia, where about 1 million new cases are identified annually and 1 billion people may be at risk for this disease. Mortality occurs in 30% to 50% of untreated cases[1]. Diagnostic techniques like IgM ELISA and IgM IFA help in early diagnosis and treatment. Though, IFA is the serological gold standard[2], IgM ELISA have remained the mainstay of diagnosis in various laboratories as it provides an objective result and has sensitivity similar to IFA[3]. Serological tests are usually negative in the first week of fever due to scrub typhus. Role of PCR for diagnosis of scrub typhus in first week for early institution of therapy was evaluated as a rapid diagnostic test. Nested and the non nested PCR formats using various gene targets have been described for the diagnosis of scrub typhus. Molecular detection using PCR is possible for skin biopsy, lymph node aspirate or EDTA blood. Real time PCR assays are as sensitive as conventional PCR, but are more rapid and give quantitative results[4]. The success of a test in confirming the diagnosis of scrub typhus is dependent on the type of sample taken and the timing of specimen[5].

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new genotypes in Himachal Pradesh[6]. In addition, quantitative real-time PCR assays to detect the 47kDa gene[7], 16S Rrna[8], 56kDa gene[1] and groEL gene[9] have been assessed for their efficiency in detection of *O. tsutsugamushi* DNA and measurement of bacterial load.

MATERIAL AND METHODS

Informed consent was obtained from all the patients or their guardians. Ethical clearance was taken from the institute's ethical committee.

Samples

Five ml of venous blood (3ml serum tube+ 2ml EDTA tube) under all aseptic conditions was collected from 327 patients clinically suspected of scrub typhus from June 2015 to May 2016.

Serology

Serum was separated by centrifugation at 2500g for 10 min and the clots were stored at -20˚C till further analysis. The serum samples were tested for IgM ELISA (In Bios Inc. USA) and IgM IFA. A titer of 0.468 OD at 465 nm for IgM ELISA and ≥64 units for IgM IFA were considered positive results.

PCR

DNA was extracted from 200 µl of plasma using the AuPreP GENbt DNA Extraction kit as per manufacturer's instructions. The purified DNA was aliquoted and amplification was carried

out by real- time format. The primers used in this study were designed by Roche, Indianapolis, IN. The gene coding for 56kDa antigen of *O. tsutsugamushi* was amplified by PCR and the primer sequences used are given below:

Forward primer: 5'-
AACTGATTTTATTCAAACTAATGCTGCT-3'.
Reverse primer: 5'-
TATGCCTGAGTAAGATACRTGAATRGAATT-3'.
Probe: 6FAM-
TGGGTAGCTTTGGTGGACCGATGTTTAATCT-TMR.

According to $N_1V_1=N_2V_2$, the volume of master mix used per reaction was 10 µl. Forward and reverse primer 1 µl each, Probe 0.8 µl and distilled water 2.2 µl per reaction to make a total volume of 15 µl. To 15 µl volume, 5 µl of the extracted DNA was added to make a total reaction volume of 20 µl. The PCR profile was determined with the following steps: Denaturation at 95˚C for 10 min followed by 40 cycles of 95˚C for 15 s and final incubation at 65˚C for 1min in a thermocycler. The assay was run on StepOne Real-Time PCR System along with a positive and negative control. The Ct value was determined for the samples positive for PCR.

Statistical analysis

IgM Micro-IFA is the reference gold standard[2]. We computed the sensitivities and specificities, the positive and negative predictive values for all the 3 tests. Statistical analysis was done using statistical software Epi-info version 7(7.1.1.0).

RESULTS

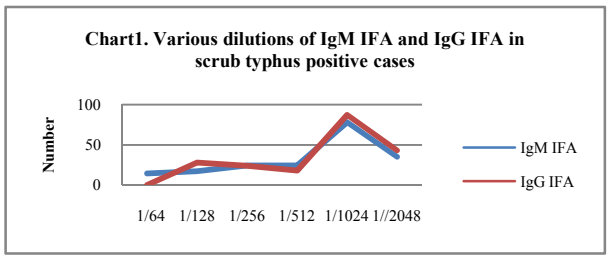
Total of 327 cases were clinically suspected of scrub typhus. 21- 60 years was the most commonly affected age group with predominance of positive cases in females of reproductive age group. Fever >39˚C was seen in all 236 patients. The duration of the fever at the time of diagnosis ranged from 5-20 days with mean duration of 12 days. Rash was seen in 35% (82/236) cases. The rash was mostly maculopapular and was predominantly found on the trunk. Eschar was seen in 33(14%) cases with chest and extremities being the most common location. Lymphadenopathy was reported in 84(35%) cases. Pulmonary manifestations were noted in 148(62.7%) cases with most patients presenting with cough 100(42%). Two cases (1%) progressed to Acute Respiratory Distress Syndrome (ARDS).

Table 1 Description of results obtained by tests used in diagnosis of scrub typhus (n=327)

Positive status category	Number of samples(%)
All positive	17(5.1)
IgM ELISA positive	177(54.1)
IgM IFA positive	174(53.2)
IgM ELISA and IgM IFA positive	174(98.3)
IgM ELISA and PCR positive	17(9.6)
All negative	157(48)

IFA for the diagnosis of scrub typhus

The baseline titre for IgM IFA and IgG IFA was calculated using 200 samples of healthy blood donors and dilutions were made from 1:64 to 1:2048. Based upon this, the cutoff value were taken as 64 and 128 for IgM and IgG IFA respectively. Total of 174 patients were positive by IgM IFA. False positivity was observed in 3 IgM ELISA cases, negative by IgM IFA



PCR for the diagnosis of scrub typhus

Out of 327 cases suspected of scrub typhus, 177(54.1%) cases were positive by IgM ELISA. PCR for 56kDa TSA was performed on IgM ELISA positive cases. Out of 177 cases who presented with fever duration of 7 or less than 7 days, 17(9.6%) were positive for PCR. The mean duration of fever in PCR positive cases was 6 days with a minimum of 1 day. Eschar was positive in only 1 PCR positive case. Most common presentation in PCR positive cases was cough with chest pain along with increased transaminase and alkaline phosphatase levels. All the PCR positive cases responded to treatment except 1 who died. The positive and negative predictive values were 100% and 35%, respectively.

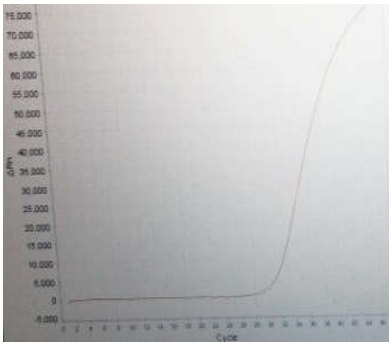


Fig 1 PCR Positive Sample

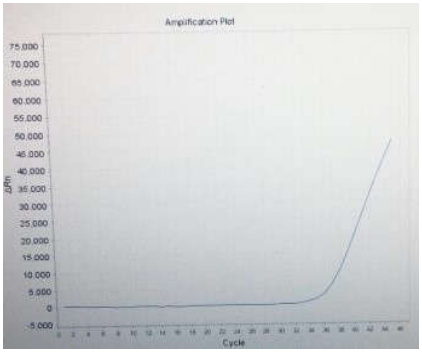


Fig 2 PCR Positive Control

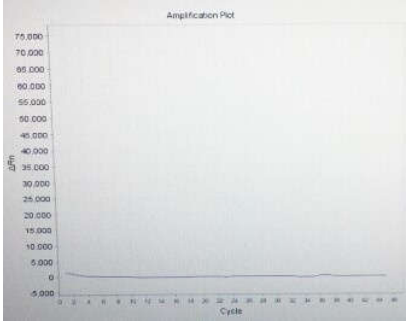


Fig 3 PCR Negative Control

Table 2 Clinical features and profile of PCR positive cases

Case No.	Fever duration	Clinical features	Biochemical abnormality	Specific antibiotic before diagnosis
27	5	Petechial Rash		Nil
62	7	Splenomegaly,Pulmonary manifestation	Thrombocytopenia, Increased transaminase and alkaline phosphatase levels	Doxycycline
63	4	Pulmonary manifestation,oliguria	Leucocytosis, Increased transaminase and alkaline phosphatase levels	Doxycycline
70	8	Hepatomegaly,Pulmonary manifestation	Anemia, Thrombocytopenia, Increased alkaline phosphatase levels	Doxycycline
84	1	Splenomegaly, oliguria	Thrombocytopenia, Increased transaminase levels	Doxycycline
90	7	Pulmonary manifestation	Thrombocytopenia	Doxycycline
100	6	Pulmonary manifestation	Leucocytosis,Thrombocytopenia	Azithromycin
106	1	Eschar,Hepatomegaly, Pulmonary manifestation	Anemia, Increased alkaline phosphatase levels	Doxycycline
108	5	Macular rash, Pulmonary manifestation	Anemia, Thrombocytopenia,Increased alkaline phosphatase levels	Doxycycline
109	9	Pulmonary manifestation	Anemia, Thrombocytopenia, Increased transaminase levels	Nil
110	8	Pulmonary manifestation	Leucocytosis, Increased transaminase levels	Nil
111	15	Rash	Anemia,Leucocytosis,Thrombocytopenia, Increased alkaline phosphatase levels	Nil
140	9	Ascitis	Leucocytosis, Increased transaminase levels	Doxycycline ,Died
142	6	Pulmonary manifestation ,Ascitis	Thrombocytopenia, Increased alkaline phosphatase levels	Nil
220	3	Hepatomegaly,Pulmonary manifestation	Increased transaminase and alkaline phosphatase levels	Doxycycline
225	3	Pulmonary manifestation	Anemia, Thrombocytopenia, Increased transaminase and alkaline phosphatase levels	Doxycycline

The sensitivity of IgM ELISA and Micro- IFA IgM was found to be 75% and 81%. The specificity of both the tests was 100%. The sensitivity of IgM ELISA as compared to IgM IFA was 95%. The specificity of IgM ELISA as compared to IgM IFA was 93%. The sensitivity and specificity of nested PCR was 9.6% and 100% respectively.

Table 3 Comparative analysis of sensitivity, specificity, positive predictive value and negative predictive value for various tests

TESTS	IgM ELISA	IgM IFA	PCR
SENSITIVITY	75%	81%	9.6%
SPECIFICITY	100%	100%	100%
PPV	100%	100%	100%
NPV	61%	67.4%	35%

DISCUSSION

The diagnosis of scrub typhus has traditionally been based on the assessment of the antibody titer in the serum samples obtained during the acute and convalescent phases of illness. However, it takes several weeks to confirm the diagnosis through serologic testing for establishing a ≥4-fold rise in titer. Antibody levels reach detectable levels by day 7 of the onset of fever. PCR is a sensitive technique to detect the gene coding for 56kDa antigen and has been used by many workers as this gene is found only in *O. tsutsugamushi* and variations in the same are responsible for the antigenic and genetic diversity of *O. tsutsugamushi*[10].

The results of PCR are available within 2-3 hours, and this can greatly help to guide proper patient management. Plasma was used to perform quantitative detection by PCR. PCR assay was based on specific primers derived from the 56-kDa major outer membrane protein antigen of *O. tsutsugamushi*. The results of PCR showed sensitivity of 9.6%, specificity of 100%, positive predictive value of 100% and negative predictive value of 35%. PCR assays, either conventional or real-time, targeting the 56 kDa gene, 47 kDa gene, *16 S rRNA* and *groEL* gene have been explored and reported to have specificity approaching 100%[10]. Sensitivity of the nested PCR assays using 56 kDa or the *16 S rRNA* genes can be as low as 22.5% to 36.1%[11].

Real-time PCR assays show a better sensitivity ranging from 45%[12] to 82%[13].

It's been shown in a study from Thailand that *O. tsutsugamushi* DNA can to be detected by the nested PCR technique as early as day 3 of the fever phase; this is before the appearance of specific antibodies in the blood[14]. However, in our study, *O. tsutsugamushi* DNA could be detected in the blood by PCR as early as the first day of fever onset. Kim *et al*. retrospectively evaluated the accuracy of conventional PCR targeting the 16S rRNA gene (16S C-PCR) for diagnosing scrub typhus and found an increased sensitivity of 87.0% and specificity of 100% compared with those obtained with other targets and is thus a simple and clinically useful method with good diagnostic accuracy.[15] In a study conducted by Prakash JAJ *et al*, only 9(10.3%) samples tested positive both for IgM ELISA and Nested PCR with a sensitivity of 58% and 100% specificity by LCA analysis[16]. The low yield in PCR could be due to the presence of heme, a known inhibitor of PCR in the sample[17]. The improved sensitivity obtained by Kim *et al*, also could be due to the volumes (50µl) used for extracting DNA[18] in contrast to 200 µl used in our study, as recommended by manufacturer. High rate of false negative PCR results whose IgM titers were high may be due to clearance of bacteria by the immune system[19]. The use of 2 sets of primer for 56kDa antigen detection in cases of Nested PCR has improved specificity.

To conclude, good specificity and low sensitivity of PCR using plasma as sample needs further evaluation of this test using blood, serum, buffy coat and eschar biopsy samples for optimizing the type of sample required for PCR. New technique for DNA extraction like Magnetic bead extraction may be evaluated comparing the conventional methods of DNA extraction for PCR. Since antigen detection tests have low sensitivity/ specificity and require biopsy specimens, in the clinical setting, serological assays are the mainstay of diagnosis[10] as they are simple and easy to perform[3].

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