Original Research Article

Performance Analysis and Evaluation of Quantitative Real Time PCR for Diagnosis of Scrub Typhus in North-East India

ABSTRACT

Aims: Scrub typhus is a life-threatening infectious disease and always creating a diagnostic dilemma in terms of rapid turnaround time and accuracy, qRT PCR can become a very good option to achieve the desired result. This study was performed to evaluate the performance of qRT PCR in comparison to commonly used IgM ELISA and Weil-Felix tests to diagnose scrub typhus, in North-East India.

Study design - This was a hospital based prospective study.

Place and Duration of Study: The study was conducted in the Department of Microbiology, of a tertiary care centre of North-East India over a period of 1 year (June 2019– May 2020). **Methodology:** Samples from suspected scrub typhus cases were screened by Weil-Felix test, followed by IgM ELISA for the confirmation of diagnosis. All the IgM positive samples and 20 highly suspected but ELISA

negative samples were subjected to qRT PCR.

Results: Out of 54 samples tested, 24 IgM ELISA positive samples and three IgM ELISA negative samples have shown the presence of bacterial DNA with quantification of DNA copies. It has also been observed that 21 out of 27 PCR positive samples (77.8%) were detected within the first seven days of illness.

Conclusion: The performance of the commercial qRT PCR kit used in our study is satisfactory, which provides the extra advantage of quantification of DNA copies and increases diagnostic accuracy within the first week of fever.

Keywords: *Scrub typhus; qRT PCR; Quantitative PCR; real-time PCR; IgM ELISA; North-East India*

1. INTRODUCTION

Scrub typhus is a life-threatening infectious disease, which represents a good share of acute undifferentiated fever mainly in the tropical region. The term “Scrub” was used due to the vegetarian terrain in which the vector – trombiculid mites reside and the word “Typhos” means ‘stupor caused by a fever’ in Greek(1). The World Health Organization (WHO) has already named scrub typhus as one of the world’s most underdiagnosed/underreported diseases which often warrants hospital admission due to acute febrile illness along with multi-organ failure(2). Scrub typhus is prevalent in many parts of India, but specific data is

not available. It is grossly underdiagnosed due to its non-specific clinical presentations, limited awareness, and low index of suspicion among physicians. Differentiating scrub typhus from other forms of typhus as well as from other causes of acute fever is often difficult during the first several days due to vague clinical presentations.

The mainstay of diagnosis of scrub typhus is serological testing. Nonspecific Weil-Felix test is extensively used everywhere but it lacks in both sensitivity and specificity. Immunochromatographic card test (ICT) is also a very popularly used tool, which also lacks in specificity. A more standardized methodology is the ELISA, which has been used for years in surveillance studies, where large number of sera can be assayed at one time. Recent nucleic acid-based techniques like conventional PCR, nested PCR and Real time PCR are useful for early and specific diagnosis.

Because of the low index of suspicion, nonspecific signs and symptoms, emergence in non-endemic areas as well as re-emergence in previously endemic areas and absence of widely available sensitive and specific diagnostic tests, Scrub typhus is really imposing a diagnostic challenge. Hence, this study was planned to evaluate the diagnostic performance of quantitative real time PCR to detect the presence of *Orientia tsutsugamushi* in patients with febrile illness, presenting to a tertiary care hospital of North-East India, where Scrub-Typhus is highly endemic.

2. MATERIAL AND METHODS

2.1 Study design - This was a hospital based cross-sectional study conducted in the Department of Microbiology, of a tertiary care center of North-East India over a period of 1 year (June 2019– May 2020). The study was conducted following the Standards for Reporting Diagnostic Accuracy (STARD) guidelines, checklist of the same is provided in table Supplementary Table 1.

2.2 Inclusion Criteria –

Case - Patients are included in the study if they have -

1. Febrile illness (fever > 37.8^{o}C) of any duration and a clinical suspicion of scrub typhus, presenting to the department of Medicine and Pediatrics.
2. Given written informed consent to participate in the study, either by themselves or by parents/guardians (for pediatric age group).

Controls – Twenty volunteers with no history of fever and no history of scrub typhus before, otherwise healthy were also included in the study as controls, after obtaining their consent.

2.3 Exclusion Criteria – Patients were excluded if a definitive diagnosis of malaria, enteric fever, tuberculosis, any other infectious or non-infectious aetiology was made during patient work-up.

2.4 Sample size – One hundred patients with acute febrile illness, without any definitive diagnosis and clinical suspicion of scrub typhus were included in the study.

2.5 Sample collection – Blood samples were collected in plain vacutainers (without anticoagulant) from 100 consecutive patients of acute febrile illness (cases) and from 20 healthy volunteers (controls), taking both inclusion and exclusion criteria under consideration.

Samples were further processed and analysed for the detection of *Orientia tsutsugamushi*.

2.6 Collection of serum samples and storage - Serum had been collected from plain vials of clotted blood after centrifuging at 2000 RPM for 10 minutes and then separating serum without disturbing the leukocyte layer.

Aliquots were made from serum samples into 3 parts for different purposes. Part 1 - For screening by Weil-Felix test, Part 2 – For doing ELISA to detect IgM against *Orientia tsutsugamushi* and Part 3 – For DNA extraction from the serum samples and PCR.

Part 2 and 3 of aliquots were stored at 2 to 8^{o}C for 48 hours, then at -20^{o}C for 1 month and for further storage of >1 month aliquots were stored at -80^{o}C.

2.7 Screening by Weil-Felix test – All the serum samples were screened first for scrub typhus by using Weil-Felix test. **"PROGEN"** Proteus antigen suspensions by Tulip Diagnostics (P) LTD. India, were used for Weil-Felix test by semi-quantitative slide method following the exact manufacturer's instructions and titers of OXK antigen were obtained.

2.8 IgM ELISA – "Scrub Typhus *Detect*^{TM} *IgM* ELISA System" by INBIOS International, Inc. was used for performing ELISA to detect IgM antibodies against *Orientia tsutsugamushi* derived recombinant antigen in stored serum samples.

2.8.1 ELISA procedure - All the serum samples from 20 healthy volunteers and all the 100 serum samples from suspected scrub typhus patients were subjected to IgM ELISA for scrub typhus following the manufacturer's instructions exactly. All the ELISA readings were obtained by using "Merilyzer EIAQuant Microplate Reader" – by Meril Life Sciences.

2.8.2 Cut-off calculation – Cut off was calculated as per the manufacturer's instructions by adding the mean of OD plus three times the Standard Deviation (SD) of normal human serum. Serum samples from 20 healthy volunteers were used to calculate the cut-off as per the manufacturer's instructions. The mean and SD of all healthy volunteers corresponded to OD values of 0.1445 and 0.0437, respectively. Using the manufacturer's cut-off formula of the final ELISA cut-off corresponded to an OD of 0.2756.

Interpretation of the results was done by following the below-mentioned criteria –

1. Samples with spectrophotometric readings > Cut off are “Reactive” and samples below this criterion are considered to be “Non-Reactive”.

2. Values near the Cut off are doubtful and the assays were repeated

2.9 DNA extraction – From all the IgM ELISA reactive samples as well as 20 highly suspected (based on clinical findings like fever, rash, loss of consciousness, respiratory distress, renal failure, not fitting to any other diagnosis) but ELISA negative samples, DNA was extracted using “QIAamp DNA Mini Kit” (Qiagen, Germany) as per the manufacturers' instructions. Extracted DNA samples were kept frozen at −80°C till the time of performing PCR.

2.10 Real time Polymerase Chain Reaction – Extracted DNA samples were subjected to quantitative real-time PCR by using “Geno-Sen's Scrub Typhus (Rotor Gene) real time PCR kit”, Genome Diagnostics Pvt. Ltd, Solan, Himachal Pradesh, India, which can detect up to (sensitivity) 70 Copies/ml of DNA. Along with detection, quantification of bacterial DNA was also done.

2.10.1 Principle of Real-Time PCR kit used -

The kit targets 56 kDa Type Specific gene of *O. tsutsugamushi*.

The primer details are as follows:

- OtsuF: 5’-AATTGCTAGTGCAATGTCTG-3',
- OtsuR: 5’-GGCATTATAGTAGGCTGAG-3'
- Accession no: KP334159.1

Real-Time PCR was performed using *RotorGene*™ Q (QIAGEN Real-time PCR Cycler)

2.10.2 Quantitation -

The quantitation standards provided in the kit (SCRUB TYPHUS S 1-5) were to generate a standard curve in the RotorGene™ Q system. The standard curves generated were used for quantitation in subsequent runs, provided that at least one standard was used in every run. The standards are defined as copies/µl. The following formula was applied to convert the values determined using the standard curve into copies/ml of sample material:

$$\text{Result (Copies/ml)} = \frac{\text{Result (Copies/µl)} \times \text{Elution Volume (µl)}}{\text{Sample Volume (ml)}}$$

2.11 Statistical analysis – The data were collected and recorded using MS-Excel for Windows v2013®. The basic descriptive statistics, frequency charts graphs, trend analysis and related graphs were computed using the same. Summary statistics and ROC curve analysis were done using MedCalc® v17.9 for Windows (MedCalc Software, Acacialaan 22, B-8400 Ostend, Belgium). The comparison of single and two proportions were done using Chi-square test. The threshold for significance was considered at $p<0.05$.

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3. RESULTS

During the study period of one year, 100 patients with febrile illness were evaluated for scrub typhus (ST). All the collected serum samples were subjected to Weil-Felix for the screening and IgM ELISA for confirmation of ST. All the IgM ELISA positive samples and 20 highly clinically suspected samples that were IgM ELISA negative were subjected to qRT PCR. Based on IgM ELISA and PCR findings 37 patients were diagnosed to be having ST, according to Scrub Typhus Inclusion Criteria (STIC criteria)(3). Other patients were followed up for the alternate final diagnosis, based on other laboratory investigation findings done by the respective departments. Different etiologies of fever other than ST were sepsis (n=17), malaria (n=4), enteric fever (n=4), tubercular meningitis (n=4), Japanese encephalitis (n=3),

pneumonia (n=1), meningitis (n=1) and dengue (n=1). In few patients (n=29) any diagnosis could not be established as some of them lost to follow up due to death before any established diagnosis or discharge against advice or non-admission as an outpatient.

Methodology of the study is depicted in Figure .1

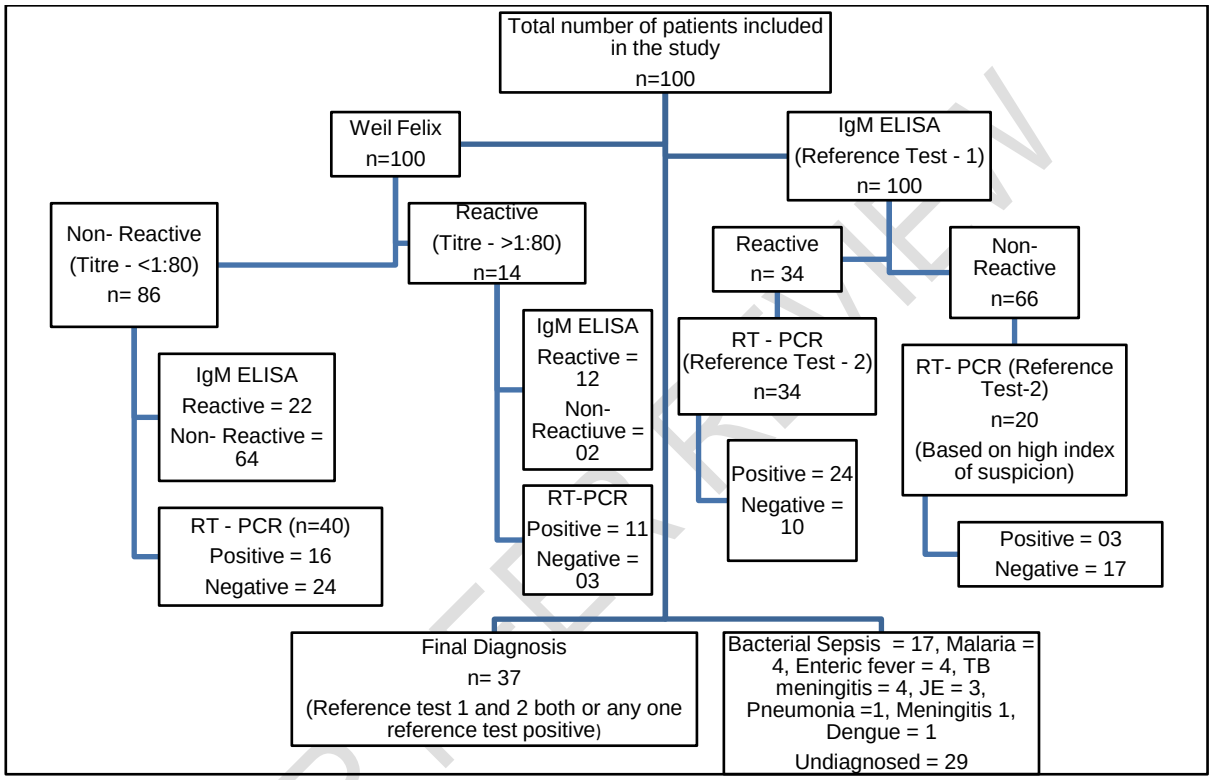


Figure1. Study methodology with results

The diagnostic profile of all the 37 diagnosed ST patients is described here on.

3.1 Weil-Felix test: Out of 100 serum samples tested only 14 samples showed high titers against OXK antigen (≥80).

Different OXK titers of all the 100 samples were - < 1:40 (n=78), 1:40 (n=08), 1:80 (n=06), 1:160 (n=04), 1:320 (n=04).

3.2 IgM ELISA for scrub typhus: Out of 100 serum samples tested, 34 samples turned out to be IgM ELISA positive among which 14 samples have shown high OXK titer (≥80), only 12 samples turned out to be IgM ELISA positive. However, out of the 86 OXK negative (titer≤ 40) samples, 22 samples were IgM ELISA positive.

3.3 Quantitative Real-Time PCR: Out of the 34 IgM ELISA positive samples 24 (70.6%) samples were positive for RT-PCR and out of 20 highly suspected but IgM ELISA negative samples three were RT-PCR positive. So, out of 54 samples tested for PCR, 27 (50%) samples were PCR positive.

Different loads of DNA copies/mL from positive PCR samples were categorized into 4 different categories and correlated with the duration of fever. Duration of fever in the patients varied from day 1 to day 14 at the time of their presentation. Day of presentation were divided in two categories ≤7 days (early presentation), 8-14 days (late presentation). DNA copies were categorized based on multiplies of 10 (Table 1).

Very low count – DNA copies in multiplies of 10^3

Low count – DNA copies in multiplies of 10^4

Medium count – DNA copies in multiplies of 10^5

High count – DNA copies in multiplies of 10^6

Table 1: Relation between DNA copies and days of fever when the sample was collected

Days of Fever	DNA copies/mL			
	Very low (No. of samples)	**Low (No. of samples)**	**Medium (No. of samples)**	**High (No. of samples)**
≤7 days	4	6	2	9
8-14 days	2	4	0	0

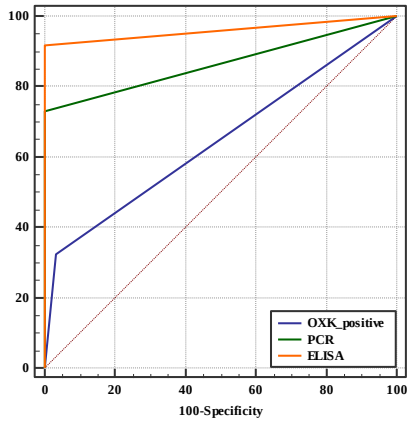
It was found that high viral count was present in samples collected within seven days of illness.

Taking IgM ELISA as the standard methodology for diagnosing scrub typhus other two tests Weil-Felix and Real-time PCR test results were compared and analyzed statistically by using different tools. Results are as follows (Table2 and Figure 2):

Table 2: Diagnostic accuracy of Weil-Felix and PCR in comparison to IgM ELISA

Diagnostic tests	Sensitivity	Specificity	95% Confidence Interval	Positive Predictive value	Negative Predictive value	Positive likelihood ratio	Negative likelihood ratio
Weil -Felix	35.3%	96.9%	0.56-0.75	85.71%	74.42%	11.65	0.67
PCR	70.59%	95.45%	0.74-0.89	88.89%	86.3%	15.53	0.31

Figure 2: Comparison of ROC curves of all the 3 tests



Variable	AUC	95% CI
OXK_positive	0.646	0.544 to 0.739
PCR	0.865	0.782 to 0.925
ELISA	0.959	0.900 to 0.989

4. **DISCUSSION**

Scrub typhus is one of the most important causes of acute febrile illness, taking a toll on a lot of lives mainly due to its initial vague presentations but serious complications, if it remains undiagnosed and not treated on time. Most of the time the disease remains undiagnosed due to non-specific clinical presentation and low index of suspicion among physicians. Presently many studies and case reports on scrub typhus are coming out from different parts

of the country, revealing new pockets and true scenarios of this dreaded disease. PCR as a molecular test score more in the aspect of sensitivity and specificity but warrants more stringent conditions. Till now most of the studies have used Nested PCR, which has shown good sensitivity and specificity but currently, Real-Time PCR is on the trends with a boon of quantification. This study was a venture to evaluate Real-Time Quantitative PCR as an accurate, less labor intensive and with an extra benefit of quantification; for diagnosing an important cause of acute febrile illness - Scrub Typhus.

In our study only 14% of patients had a positive OXK titer of ≥80. Considering this titer of ≥80 we found that, the sensitivity of the test is only 35.29% and specificity of 96.97%. Weil-Felix test has shown a positive predictive value of 85.7% and a negative predictive value of 74.42%. The positive Likelihood Ratio for the Weil-Felix test was 11.647 and the Negative Likelihood Ratio was 0.667.

In a study by Prakash *et al*., conducted at Vellore,Tamilnadu; Weil-Felix test has shown a sensitivity of 59% and similar specificity of 94% when cutoff titer of ≥80 was used(4). Another study by Anitha *et al*. performed at Puducherry has reported the sensitivity of Weil-Felix as 81.34%, 65.67%, and 50.38%, whereas the specificity of 70.93%, 94.19%, 95.51% with OXK titers of 80, 160 and 320, respectively(5).

In a resource-poor set-up like India, especially in the corner most part of the country – the north-eastern region, easy to do and cheaper ways of diagnostics will always be more preferred. Therefore, in an endemic zone of scrub typhus, Weil-Felix will be a preferred tool for screening and its results can be confirmed by serological tools like IgM ELISA or Molecular tests like Real-time PCR. The sensitivity of a test suffers a lot if high cut off titers of antibodies against OXK is used. Therefore, the seroprevalence of scrub typhus and thereby determining the cut off titer for OXK is especially important. Moreover, Weil-Felix has a good PPV and NPV despite having low sensitivity as shown in our study. So, it remains a good tool for the screening of scrub typhus.

As Immunofluorescence assay (IFA) – the gold standard test for scrub typhus, was not available and moreover its lack of standardization worldwide(6), IgM ELISA was considered as the standard methodology test in our study. All the 34 positive samples by IgM ELISA were true positive samples and were used to compare the results of the other two tests.

In a recent study to evaluate the diagnostic accuracy of the InBios Scrub Typhus Detect ELISA kit for the detection of IgM Antibodies by Blacksell *et al*., which was also used in our study, a sensitivity of 93% and specificity of 91% was reported when cut off OD value for IgM ELISA was calculated according to manufacturer's instructions(7).

Jang *et al*. evaluated IgM ELISA for the diagnosis of scrub typhus and reported sensitivity of 100% and specificity of 99% for IgM IFA-positive samples(8). The performance and

diagnostic accuracy of this IgM ELISA kit were validated by different authors in India (5,9–13) and the results were quite satisfactory. Even though IFA is the gold standard test for diagnosing scrub typhus, it is difficult to perform, cumbersome requires a skilled observer, mandates inclusion of several antigenic types, is subjective and is too expensive to be imported(13). The sensitivity and specificity of ST IgM ELISA are almost equivalent to those of IFA, and it can be performed by most laboratories(14,15). Therefore, for confirmation of the screening positive samples, IgM ELISA is a very good alternative to IFA.

However, few false-positive results were obtained by Prakash *et al.* conducted at Vellore, in patients with falciparum malaria, pulmonary tuberculosis, *Streptococcus viridans* septicemia, and typhoid fever(10). But we in our study did not get any such instances of cross-reactivity.

When Real-time PCR results were analyzed statistically, it has shown a sensitivity of 70.59%, specificity of 95.45%, a positive predictive value of 88.89% and a negative predictive value of 86.3%. It has also shown a positive likelihood ratio of 15.529 and a negative likelihood ratio of 0.308.

In a similar study by Anitharaj *et al.,* evaluation of the same real-time PCR kit was done which was used in our study, at JIPMER Puducherry. In that study 49(38.8%) samples were positive out of 126 samples tested. Out of 77 ELISA positive samples, 42 (54.54%) were PCR positive. In that study, the sample of choice was a buffy coat and IgM ELISA was used as an alternative to IFA for serological confirmation, which was like our study(16).

The difference between positivity rates amongst both the studies can be attributed to the different prevalence rates of the study location.

In another study by Singhsilarak *et al*. 17 (62.9%) samples were positive by quantitative real-time PCR out of 27 samples, confirmed serologically by IgM ELISA, which is like our findings(17).

Another study was done by Bakshi *et al*. at Puducherry, to develop a qualitative real-time PCR targeting the 56kDa gene. In that study, 73% of samples turned out to be PCR positive amongst the clinically suspected and OXK positive (cut off titer ≥160) serum samples(18).

Molecular diagnosis always takes the upper hand from serological tests, as using PCR tests *Orientia* DNA can be detected accurately and within the 1st week of infection before the appearance of antibodies in the human blood. Over the year's different types of PCR assays like conventional, nested, qualitative real-time PCR assays were tried and evaluated for the diagnosis of scrub typhus. Recently quantitative real-time PCR kits are being used in India, "Geno-Sen's Scrub Typhus (Rotor-Gene) real-time PCR kit" is one of those. The target gene in this kit is 56-kDa, which is the most common targeted gene. According to the manufacturers, the specificity of the kit is 100% and sensitivity is remarkably high too, having the ability to detect DNA load of as low as only 70 copies/mL.

In our study, 10 IgM ELISA positive samples were PCR negative. These 10 samples cannot be ignored as false ELISA positive, as detection of 56 kDa gene depends on the duration of febrile illness at the time of blood collection and samples used. Eschar samples are found to be yielding better results for PCR(19). Usually, ST-qPCR gives positive results within the 1st week of the acute illness, whereas IgM antibody starts appearing from day seven onwards. In our study 21(77.8%) out of 27 PCR samples were collected within the 1st seven days of illness rest other six samples were obtained in the 2nd week of illness. According to Saisongkorh *et al*., ST-PCR positivity was observed up to day 22 in some patients(20). Compared to other types of PCR, real-time PCR using the above-mentioned kit is faster – results can be obtained within two hours. Another advantage of quantitative real-time PCR is its quality of quantification. This added advantage can be utilized for knowing the disease severity and treatment follow-up. Being a test with good sensitivity and specificity and better PPV and NPV values, qPCR can be used as a particularly good confirmatory test. Though cost-effectiveness and requirement of stringent conditions for sample processing and testing make this test unacceptable to be used as a routine test.

4.1 Limitations of the study – i) Other than serum samples buffy coats, blood clots or eschar samples could have been used for DNA extraction to get a better yield of extracted DNA. ii) Only one gene was targeted in the used PCR kit, whereas the other two genes, namely 47kDa and groEL genes were not considered. This can be related to the lower sensitivity when compared with IgM ELISA positive samples. iii) ST IFA test was not done which is considered to be the “Gold Standard” test for diagnosing scrub typhus. This can be attributed to the lack of availability of the IFA kit in India and the lack of complete evaluation. Moreover, the technical glitch and subjectivity of the reporter’s observation makes it difficult to perform and restricts its usefulness. iv) PCR results could have been better if all the samples could have been collected on the 1st week of illness. Although it has not shown any statistical significance in our study there is always a higher chance of better yield of bacterial DNA before the appearance of antibodies. v) Sample size could not be calculated due to lack of prevalence data and the study was restricted to only 100 samples due to resource constrains and feasibility of the study.

5. CONCLUSION

In a resource constraint place like North-East of India, Weil-Felix can still serve the purpose of screening out the cases of scrub typhus but for confirmation IgM ELISA or Real-Time

PCR can be used. If we can combine the power of both the tests then there will be less chances of missing a case.

The performance of the commercial quantitative real-time PCR kit used in our study is satisfactory. Molecular diagnosis should be carried out preferably during the 1st week of febrile illness for better results. Due to the reasonable levels of sensitivity and specificity of Real-time PCR, its continued usage as a standard methodology in developing countries like India may be a good choice, as utility and availability of IFA kits are still questionable and culturing *Orientia* is a tough nut to crack.

CONSENT

All authors declare that 'written informed consent was obtained from the patient (or other approved parties) for publication of this study and accompanying images. A copy of the written consent is available for review by the Editorial office/Chief Editor/Editorial Board members of this journal."

ETHICAL APPROVAL

Ethical approval was taken from the institutional Ethics Committee, letter no. - NEIGR/IEC/2019/0040.

COMPETING INTERESTS DISCLAIMER _

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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APPENDIX

Supplementary Table 1

Section & Topic	No	Item	Reported on page #
TITLE OR ABSTRACT		Performance Analysis and Evaluation of Quantitative Real Time PCR for Diagnosis of Scrub Typhus in North-East India	1, (Lines 1-3)
	1	Identification as a study of diagnostic accuracy using at least one measure of accuracy (such as sensitivity, specificity, predictive values, or AUC)	10,11 (Lines 185,188)
ABSTRACT			
	2	Structured summary of study design, methods, results, and conclusions (for specific guidance, see STARD for Abstracts)	1,2
INTRODUCTION			
	3	Scientific and clinical background, including the intended use and clinical role of the index test	3,4 (Lines 24-43)
	4	Study objectives and hypotheses	4 (Lines 44-50)
METHODS			
Study design	**5**	Whether data collection was planned before the index test and reference standard were performed (prospective study) or after (retrospective study)	4 (Lines 54-58)
Participants	**6**	Eligibility criteria	4-5, (Lines 59-69)
	7	On what basis potentially eligible participants were identified (such as symptoms, results from previous tests, inclusion in registry)	4 (Lines 59-66)
	8	Where and when potentially eligible participants were identified (setting, location and dates)	4 (Lines 54-56)
	9	Whether participants formed a consecutive, random or convenience series	5 (Lines 73)
Test methods	**10a**	Index test, in sufficient detail to allow replication	5-6 (Lines 85-88, 106-130)
	10b	Reference standard, in sufficient detail to allow replication	5-6 (Lines 89-105)
	11	Rationale for choosing the reference standard (if alternatives exist)	12 (Lines 239-244)
	12a	Definition of and rationale for test positivity cut-offs or result categories of the index test, distinguishing pre-specified from exploratory	6 (Lines 96-101)
	12b	Definition of and rationale for test positivity cut-offs or result	6 (Lines 102-

		categories of the reference standard, distinguishing pre-specified from exploratory	105)
	13a	Whether clinical information and reference standard results were available to the performers/readers of the index test	-
	13b	Whether clinical information and index test results were available to the assessors of the reference standard	-
Analysis	**14**	Methods for estimating or comparing measures of diagnostic accuracy	10-11 (Lines 183-196)
	15	How indeterminate index test or reference standard results were handled	-
	16	How missing data on the index test and reference standard were handled	-
	17	Any analyses of variability in diagnostic accuracy, distinguishing pre-specified from exploratory	-
	18	Intended sample size and how it was determined	-
RESULTS			
Participants	**19**	Flow of participants, using a diagram	9
	20	Baseline demographic and clinical characteristics of participants	-
	21a	Distribution of severity of disease in those with the target condition	8 (Lines 142-147)
	21b	Distribution of alternative diagnoses in those without the target condition	8 (Lines 148-152)
	22	Time interval and any clinical interventions between index test and reference standard	-
Test results	**23**	Cross tabulation of the index test results (or their distribution) by the results of the reference standard	10 (Table 2)
	24	Estimates of diagnostic accuracy and their precision (such as 95% confidence intervals)	10 (Table 2)
	25	Any adverse events from performing the index test or the reference standard	-
DISCUSSION			
	26	Study limitations, including sources of potential bias, statistical uncertainty, and generalisability	14 (Lines 289-300)
	27	Implications for practice, including the intended use and clinical role of the index test	14-15 (Lines304-318)
OTHER INFORMATION			
	28	Registration number and name of registry	-
	29	Where the full study protocol can be accessed	-
	30	Sources of funding and other support; role of funders	15