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Scrub typhus diagnosis on acute specimens using serological and molecular assays — a 3-year prospective study

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ABSTRACT

Scrub typhus (ST) is an underdiagnosed acute febrile illness in the Asia Pacific region with recent reemergence. Clinical diagnosis is difficult, and laboratory confirmation is largely based on serological and molecular tests. However, Weil–Felix test still remains the only test available in much of the rural tropics where a disproportionate number of cases occur. Sensitive and affordable assays are important for broader use and accurate diagnosis. We evaluated the diagnostic capabilities of serological and molecular assays on single acute clinical samples. Out of 1036 cases, 319 were confirmed as ST, and the sensitivities of immunofluorescent assay (IFA), IgM enzyme-linked immunosorbent assay (ELISA), nested polymerase chain reaction (n-PCR) and WFT were 93.4%, 80.3%, 75.2%, and 54.2%, respectively. IgM ELISA + n-PCR combination demonstrated highest degree of agreement ($\kappa = .911$) in the absence of IFA. Additionally, 16 cases were detected by n-PCR only. Our study emphasizes the diagnostic challenges in the developing world, importance of molecular tests, and best alternate assays in ST diagnosis.

1. Introduction

Scrub typhus (ST) is a vector-borne zoonotic acute febrile illness caused by the obligate intracellular bacterium, *Orientia tsutsugamushi*, and commonly seen in the tropics of rural Asia Pacific region (Tamura et al., 1995). The word "tsutsugamushi" is derived from Japanese language, "tsutsuga" meaning "illness" and "mushi" meaning "insect" (Kelly et al., 2002). The other names include tropical or rural typhus (Lewthwaite and Savoor, 1940), chigger-borne rickettsiosis, mite-borne typhus, tsutsugamushi disease, and Japanese river fever (Kelly et al., 2002). Chiggers, the larval stages of the mite, are the vectors and require a body fluid/blood meal from vertebrates for its development. Humans are the incidental and terminal host, who may acquire the infection after entering accidentally into mite-infested areas. The genus *Orientia* contains two species, *O. tsutsugamushi* (>20 distinct antigenic variants identified) (Kelly et al., 2009; Zhang et al., 2013) and "*Candidatus O.* chuto," a recently recognized species (Izzard et al., 2010). The first identified prototype strains of *O. tsutsugamushi* were Karp, Kato, and Gilliam (Shishido et al., 1969).

ST presents with undifferentiated fever with signs and symptoms similar to the other tropical febrile illness such as dengue, leptospirosis, malaria, and other viral hemorrhagic fevers, obfuscating the clinical diagnosis (Koraluru et al., 2015). Presence of an eschar is helpful; however, it is not always present, and chances of missing one are apparent if not thoroughly searched (Kim et al., 2007; Munegowda et al., 2014). Laboratory diagnosis of ST is also challenging, mainly in the rural tropics, as Weil–Felix test (WFT) still remains as the only available test owing to its simplicity (Cruickshank, 1927; Koraluru et al., 2015). Over the past

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few decades, newer diagnostic assays for rickettsial diseases have been developed but are not easily available worldwide. These newer assays were not available in developing or the underdeveloped world until recently as they are costly, need trained operators and specialized equipment, and always need to be imported (Varghese, 2014). Serological assays were the single most important test in laboratory diagnosis of ST until early 1990s. Immunofluorescent assay (IFA) is the current gold standard test in ST diagnosis. However, the difficulties include requirement of homologous antigens, variable cutoff titers across endemic countries, and subjectivity of the assay. Furthermore, serological assays are generally negative in the early stage of the illness (first week) and require a convalescent sample (10–14 days apart) to confirm the diagnosis, thereby limiting its usefulness in early and optimal selection of antibiotics. Molecular tests have become popular over the years as they can detect the disease at an earlier stage and are specific even in a single acute specimen. Recent reports downgraded the utility of WFT in ST diagnosis when compared to other tests (Kularatne and Gawarammana, 2009; Pradutkanchana et al., 1997). As relatively newer (in the developing world) and better diagnostic tests such as IFA, nested polymerase chain reaction (n-PCR), and quantitative real-time PCR (Q-PCR) are available, still using the WFT is questionable. Additionally, diagnostic usefulness of individual and combination tests in comparison to M-IFA in a statistically significant Indian population was lacking.

In this study, we evaluated the diagnostic usefulness of serological tests and a molecular test on a single acute stage clinical sample for ST in a large statistically significant Indian population. Further, to assist the diagnosis of ST in less equipped laboratories, the best assay or assay combinations were determined to aid clinical microbiologists and clinicians to transition towards better assays.

2. Methodology

2.1. Ethics statement

The study was conducted at the largest tertiary care hospital in southwestern India (Kasturba Hospital, Manipal) with a 2032-bed capacity. The study duration was from June 2012 to January 2015. Institutional Ethical Committee (IEC80/2011)) approval was obtained before the study. All the patients included in the study provided written informed consent prior to sample and history collection.

2.2. Patient samples

A total of 1036 patients admitted at Kasturba Hospital, Manipal who were suspected of having ST were prospectively recruited during the present study. Only adults patients (≥18 years of age) of both sexes who provided the written informed consent were included.

Clinical material included acute stage blood samples (4 mL of EDTA blood and 4 mL of blood in a clot activator tube for serum, and blood for blood cultures).

2.3. Serum processing

The clot activator tubes were centrifuged at 400×*g* for 10 min to separate the serum. The serum was stored at −80°C until further use.

2.4. Separation of peripheral blood mononuclear cells (PBMCs) using HISTOPAQUE® 1077 solution

PBMCs were separated using Histopaque solution, the technique based on the principle of density gradient centrifugation. Histopaque 1077 (Sigma Aldrich Chemicals, Bengaluru, India) solution contained polysucrose and sodium diatrizoate, adjusted to a density of 1.077 g/mL.

Briefly, 3mL of the Histopaque solution was placed in a sterile 15-mL graduated centrifuge tube, 4 mL of the EDTA blood was gently layered on top of the Histopaque solution, and the tubes centrifuged at 400×*g* for 30 min. The plasma layer was aspirated into a sterile cryovial, leaving a small volume behind. The PBMC layer was carefully aspirated using a sterile Pasteur pipette into a fresh 15-mL graduated centrifuge tube and washed 3 times with 10 mL of phosphate-buffered saline (PBS) with a pelleting step between washes (250×*g* for 10 min). At the end, the pellet was resuspended in 500 μL of sterile PBS and preserved at −80°C, and DNA was extracted generally the following day.

2.5. DNA extraction from PBMCs

DNA from the PBMCs was extracted using QIAamp DNA blood mini kit (Qiagen, Hilden, Germany). Two-hundred microliters of the PBMCs was used to extract DNA following the manufacturer's instructions. DNA was eluted using 200 μL of the elution buffer supplied in the kit into a 1.5-mL cryovial and preserved at −80°C until further use.

2.6. Serological and molecular tests performed to detect ST

The WFT, IgM enzyme-linked immunosorbent assay (ELISA), and IgM M-IFA (Fig. 1) were performed as previously described by Koraluru et al (Koraluru et al., 2015). n-PCR was performed according to the method previously described (Furuya et al., 1993) in which the primers amplified the 56-kDa type specific antigen gene of *O. tsutsugamushi* (Fig. 2). All the primers used in the present study were procured from Sigma Aldrich Chemicals (Bengaluru, India). Final PCR amplification

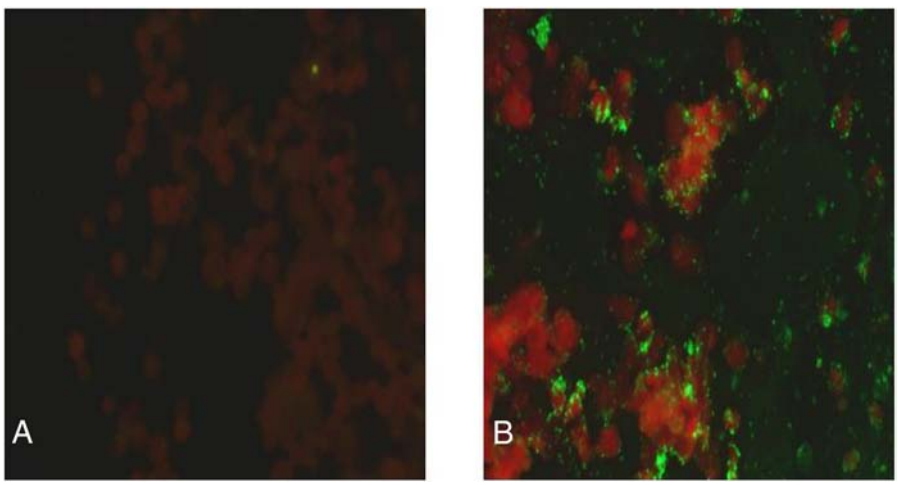

Fig. 1. (A) Absence of apple green fluorescence in a negative IFA test result. (B) Intracellular and extracellular apple green fluorescent bacilli of *O. tsutsugamushi* in a positive IFA test result.

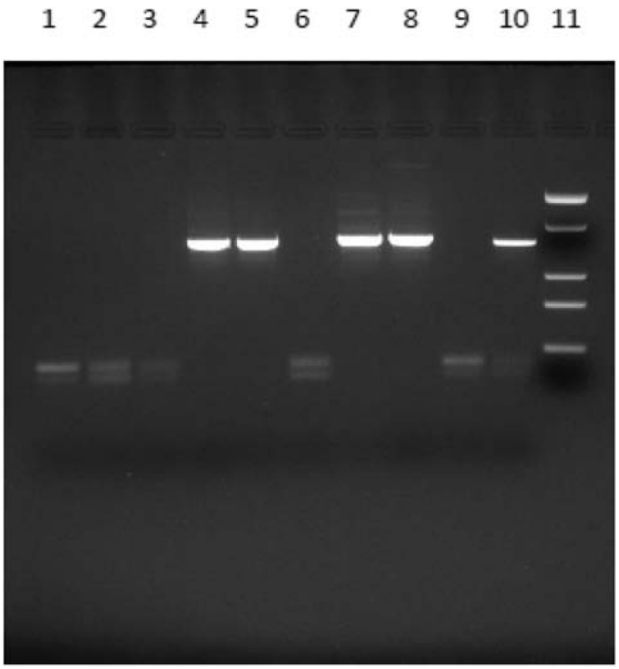

Fig. 2. Gel electrophoresis of n-PCR products on 1.5% agarose gels. Lanes 1–8 = patient samples, lane 9 = negative control, lane 10 = positive control, and lane 11 = DNA ladder (100, 200, 300, 600, and 1000 bp).

products were separated on 1.5% agarose gels, and results were documented.

2.7. Statistical analysis

The clinical and laboratory data were entered into SPSS (SPSS for Windows, Version 15.0, South East Asia, Bengaluru). For all study variables, descriptive statistics were obtained, categorical data were expressed as percentages, and continuous data were summarized using mean and standard deviation. The sensitivities and specificities of serological and molecular assays were calculated using MedCalc for Windows, version 13.3 (MedCalc Software, Ostend, Belgium). Test of association was performed using Pearson's Chi square test, and a *P* value of <0.05 was considered statistically significant. Kappa statistics were calculated to determine the degree of agreement between the assays.

2.8. Scrub typhus infection criteria (STIC) for the present study

Following case definitions were followed in the present study:

2.8.1. Probable case of ST

Positive for at least any 1 of the ST test performed.

2.8.2. Confirmed case of ST

Positive n-PCR for *Orientia*-specific 56-kDa TSA gene in PBMCs and/or positive by M-IFA at 1:128 supported by positive clinical response to antirickettsial antibiotics.

Table 1
Number of cases positive by each ST test performed.

Sl No	Test	Positive cases	Percentage (%)
1	WFT (OX-K)	218	21.0
2	IgM ELISA	285	27.5
3	M-IFA (IgM)	298	28.8
4	n-PCR (56-kDa TSA)	240	23.2

Table 2
Positivity by individual test for ST.[a]

No.	Test	Positive cases	Percentage (%)
1	WFT (OX-K ≥1:160)	173	54.2
2	IgM ELISA	256	80.3
3	M-IFA (IgM)	298	93.4
4	n-PCR (56-kDa TSA)[a]	240	75.2

[a] Sixteen cases were positive only by n-PCR.

3. Results

Of the 1036 cases studied, 368 (35.6%) were considered as probable case of ST, of which 319 (30.8%) were confirmed as ST. The number of cases positive by each ST test performed in the present study is outlined in Table 1.

3.1. Baseline demographics (n=319)

A slight male preponderance was seen among confirmed ST cases with 179 cases (56.1%) being males and 140 (43.9%) females. The median and interquartile range (IQR) for age were 40 (29.0–51.0) years for males and 40.5 (30.2–50.0) years for females. The median and IQR for duration of fever before seeking medical assistance were 8 (6–12) days in males and 8 (7–10) days in females.

The percentage positivity of M-IFA, IgM ELISA, n-PCR (56-kDa TSA), and WFT (OX-K ≥1:160) was 93.4 %, 80.3%, 75.2%, and 54.2 %, respectively (Table 2); however, in the combination assays, WFT+ IgM ELISA + n-PCR combination showed the highest positivity (97.8%) (Table 3).

3.2. Diagnostic validation

Diagnostic capabilities of individual test and combination tests were measured by determining the diagnostic parameters such as sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) and measure of agreement (κ coefficient). (Table 3). Among the individual tests, M-IFA exhibited the highest sensitivity (93.4%) with a good measure of agreement ($\kappa = .952$) followed by IgM ELISA, n-PCR, and WFT with sensitivities of 80.3%, 75.2%, and 54.2%, respectively. WFT showed specificity quite close to that of IgM ELISA, which may be due to the higher cutoff followed in the present study (Table 4).

Excluding M-IFA, the best combination assays with decreasing measure of agreement were in the order of IgM ELISA + n-PCR, n-PCR + IgM ELISA + WFT, WFT+ n-PCR, and WFT+ IgM ELISA, with a measurement of agreement of $\kappa = .911$, .878, .835, and .766, respectively. The 3-test combination (n-PCR + IgM ELISA +WFT) demonstrated a higher sensitivity of 97.8% in comparison to IgM ELISA + n-PCR 96.5%; however, the specificity was only 93.2% against 95.9%. Due to this, IgM ELISA + n-PCR combination showed better measure agreement of $\kappa = .911$ against $\kappa = .878$ by n-PCR + IgM ELISA + WFT. The least sensitivity, specificity, PPV, NPV, and measure of agreement were seen for WFT+ IgM ELISA combination, wherein both tests are serological tests (Table 5).

Table 3
Number of cases positive by combination of 2 or more tests.*

Sl No	Test/combination of tests	Positive cases	Percentage
1	WFT+ IgM ELISA[a]	265	83.1
2	WFT+ n-PCR[a]	290	90.9
3	IgM ELISA + n-PCR[a]	308	96.6
4	WFT+ IgM ELISA + n-PCR[a]	312	97.8

* Number of cases positive by any of the assay in the combinations mentioned.

Table 4
Performance of individual tests in ST diagnosis and measure of agreement (in comparison to our STIC).

Test	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	κ value
WFT (OX-K) 1:160	54.2% (48.6–59.8)	93.7% (91.7–95.4)	79.4% (73.4–84.5)	82.2% (79.4–84.7)	.526
IgM ELISA	80.3% (75.5–84.5)	95.9% (94.2–97.3)	89.8% (85.7–93.1)	91.6% (89.4–93.5)	.785
M-IFA (IgM)	93.4% (90.1–95.8)	100 % (99.5–100)	100 % (98.8–100)	97.1 % (95.7–98.2)	.952
n-PCR	75.2% (70.1–79.9)	100% (99.5–100)	100% (98.5–100)	90.1% (87.8–92.1)	.808

CI = confidence Interval; PPV = positive predictive value: NPV = negative predictive value. A good measure of agreement was seen for tests M-IFA and n-PCR.

4. Discussion

Diagnosis of rickettsial diseases including ST is more difficult than the treatment itself in the developing and underdeveloped world. Even today, WFT is still the single and most important test in rural tropics of Asia including India. Early diagnosis of ST would substantially decrease the cost management by early initiation of appropriate therapy, quicker and successful recovery, and avoiding other unnecessary investigations in both resource-poor and resource-well countries. However, inability to grow *Orientia* in conventional agar-based cultures, nonavailability of agent specific assays, and clinical difficulty in differentiation of ST from other tropical febrile illness are additional causes for underdiagnoses of ST. Complicating these is the further need to show 4-fold rise in antibody levels by serological tests for disease confirmation between acute and convalescent sera. This is generally not possible as most cases do not come for follow-ups.

4.1. WFT (OX-K)

The antigens used in WFT are the somatic antigens (O antigens) of *Proteus* spp. It was believed that the peptidoglycan and lipopolysaccharide components of *O. tsutsugamushi* cross-reacted with the WF antigens. It was identified that *O. tsutsugamushi* lacked peptidoglycan and lipopolysaccharide components (Amano et al., 1987); however, the most recent research demonstrated the presence of peptidoglycan-like structure in the cell envelope of *O. tsutsugamushi* (Atwal et al., 2017). It remains unsubstantiated what component of the *Orientia* leads to these cross-reactions. Recent reports describe the grossly insensitive and nonspecific nature of the assay and demand better assays in patient care (Koraluru et al., 2015; Kularatne and Gawarammana, 2009). In the present study, it was observed that although the antigens used in the WFT are of nonrickettsial in nature, the test is reasonably specific for the diagnosis of ST at a cutoff titer of 1:160; however, the sensitivity was not so encouraging. These findings are in concordance with previous studies (Brown et al., 1983; Pradutkanchana et al., 1997). Alternatively, many studies have also reported lesser sensitivities of WFT at a varied cutoff titers ranging from 1:20 to 1:320 and discouraged its use in routine diagnosis (Issac, 2004; Kularatne and Gawarammana, 2009; Prakash et al., 2006; Tay, 2003). Additionally, in another Indian study, WFT at a cutoff titer of 1:80 demonstrated poor sensitivity (30%) but good specificity (100%) (Issac, 2004). The heterophile nature of the WFT antigens and antigenic heterogeneity in the rickettsial species probably are the main reasons for the failure of WFT.

It is surprising to see that WFT showed good specificity but poor sensitivity (Table 3), as this is a heterophile agglutination test. The higher specificity may be due to proper determination of baseline titers in our endemic population as cutoff titer. Even then, the actual component of *Orientia* that leads to this cross-reaction between *Proteus* spp. is still uncertain. Clinicians must be mindful of the fact that a positive WFT is much more informative than a negative WFT. The probable reasons for failure of the WFT may be due to relative late appearance of the appropriate antibodies (Sayen et al., 1946), suppression of antibodies due to prior antibiotic treatments (Smadel et al., 1952), or absence due to reinfections with *O. tsutsugamushi* (Koralur et al., 2018; Smadel et al., 1952), which is a common observation in endemic areas of Asia (Bourgeois et al., 1982; Pradutkanchana et al., 1997). Although WFT is an affordable test in resource-poor countries, it lacks good sensitivity and specificity, especially at lower cutoff titers. It is imperative to gradually replace this assay with specific serological tests such as IFA or ELISA.

4.2. IgM ELISA

The usefulness of ELISA in ST diagnosis was described as early as 1979 (Dasch et al., 1979). The earlier ELISAs used antigens that were extracted and purified from rickettsia grown in host cells, which were cumbersome and noneconomical. With the advent of r-DNA technology, mass production of *Orientia*-specific cell wall components such as 56-kDa type specific antigen is possible in expression systems such as *Escherichia coli* (Ching et al., 1998). Presently, these recombinant antigens have been incorporated into ELISA and rapid flow assay test formats (Ching et al., 2001; Coleman et al., 2002).

The ELISA used in the present study was based on the combination of recombinant antigens of Karp, Kato, Gilliam, and TA 763 genotypes and observed to have a sensitivity of 80.3% and a specificity of 95.9% (Table 3). Similar sensitivity and specificity were observed in another study in the Indian population (Prakash et al., 2006). However, a study from Thailand observed better sensitivity but slightly lesser specificity

Table 5
Performance of combined assays in ST diagnosis and measure of agreement.

Test combinations	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	κ value
WFT+ IgM ELISA	83.1% (78.5–87.0)	93.2% (91.1–94.9)	84.4% (79.9–88.2)	92.5% (90.4–94.3)	.766
WFT+ n-PCR	90.9% (87.2–93.8)	93.7% (91.7–95.4)	86.6% (82.4–90.0)	95.9% (94.1–97.2)	.835
IgM ELISA + n-PCR	96.5% (93.9–98.3)	95.9% (94.2–97.3)	91.4% (87.9–94.2)	98.4% (97.2–99.2)	.911
n-PCR + IgM ELISA + WFT	97.8% (95.9–99.1)	93.2% (91.1–94.9)	86.4% (82.5–89.8)	98.9% (97.8–99.6)	.878

CI = confidence Interval; PPV = positive predictive value; NPV = negative predictive value. IgM ELISA and n-PCR combination assays detected the maximum number of cases with a good measure of agreement.

of 93.0% and 94.0%, respectively, at a cutoff titer of 1:400 (Coleman et al., 2002). Most studies reported across the endemic regions have used recombinant 56-kDa TSA of *O. tsutsugamushi* as the antigen in the ELISA, which could be the probable reason to observe close sensitivity and specificities (Ching et al., 1998; Coleman et al., 2002; Kim et al., 2013; Koraluru et al., 2015; Prakash et al., 2006). The IgM ELISA is definitely far superior to WFT and has become the test of choice in recent years in many parts of the Indian subcontinent because the tests kits are available commercially and do not require dedicated instrumentation except for an ELISA optical density (OD) reader and because of ease in interpretation of results. However, careful consideration must be given to determine the cutoff OD prior to utility of IgM ELISA in routine diagnosis at any given endemic area.

4.3. M-IFA

The indirect immunofluorescent assay for rickettsial diseases was previously described by Goldwasser and Shepard as early as 1959. Utility of the IFA in Rocky Mountain spotted fever and epidemic typhus was described in 1976 (Philip et al., 1976). Indirect immunofluorescent assay on smears of *O. tsutsugamushi* was found to be serologically diagnostic (Bozeman and Elisberg, 1963). In addition, the assay exhibited no cross-reactions among other rickettsial diseases. A modification to the existing IFA into micro format enabled simultaneous detection of specimen against 9 different antigenic strains (Robinson et al., 1976). IFA test was also possible on the capillary blood specimens collected on blotting paper (Gan et al., 1972).

Although IFA is considered the current gold standard test in serodiagnosis, it is observed that diagnostic cutoff titers vary from 1:10 to 1:400 not only between countries but also within a smaller geographical region. Due to this, there is lack of consensus on diagnostic titers, leading to confusion (Blacksell et al., 2007). Recently, a combination of culture, PCR assays and IFA IgM (STIC) was used as reference standard to evaluate alternative diagnostics (Paris et al., 2011). Such a combination of assays was not possible in our study due to nonavailability of cell culture facilities and very limited access to convalescent sera.

M-IFA detected majority (93.4%) of the ST cases, most of which were also positive by n-PCR (70.2%) and also showed dramatic clinical recovery to antirickettsial antibiotics. As there were molecular evidence, good clinical response to antirickettsial antibiotics, and significant levels of antibodies in serum with assay cutoff values determined experimentally, results on a single acute sample were considered sufficient. Among the serological tests, the better performance of M-IFA is possibly explained by the use of better antigens. The antigens used on the M-IFA slides are whole cell antigens, wherein total antibodies against all of *Orientia* proteins were detected, whereas in the r56-kDa TSA ELISA, only antibodies against the recombinant 56-kDa protein were detected. Further, incorporation of more than 1 homologous prototype antigen could explain the probable reasons for superior performance of M-IFA and IgM ELISA in comparison to nonhomologous antigens in WFT. The additional downside of IFA would be the need of a fluorescent microscope, training in interpretations, and more importantly continuous availability of antigen slides. Even with all these negatives, IFA stands out as a far superior test in ST diagnosis.

4.4. Nested PCR

Utility of the n-PCR in ST diagnosis was described as early as 1993 (Furuya et al., 1993) by the same group who standardized conventional PCR for ST diagnosis in 1991 (Furuya et al., 1991). Other common molecular strategies are Q-PCR and loop-mediated amplification assay (Huber, 2012; Jiang et al., 2004). n-PCR is known to be 100 times more sensitive over a single PCR (Murai et al., 1992), so we preferred using n-PCR as one of the molecular tests in our study for diagnostic validation.

n-PCR demonstrated a sensitivity and specificity of 75.2% and 100%, respectively. These results are in correlation with the results of Korean researchers wherein a sensitivity of 82.2% and specificity of 100% were observed using whole blood samples while comparing the performance against indirect IFA (Dong-min-Kim et al., 2006). Contradicting this finding, an Indian study reported the underperformance of n-PCR over IgM ELISA with sensitivity and specificity of 58% and 100%, respectively (Prakash et al., 2011). Researchers in this study used latent class analysis in the diagnostic validation of test as there was no gold standard test available in their study; they ruled out technical error in DNA extraction process and assumed that the probable reasons for lesser sensitivity of n-PCR in their study could be due to larger elution volumes and presence of heme. Heme is a potential inhibitor of PCR (Liu et al., 2006), and the inhibitory effect of heme in our study was circumvented by separating PBMCs using density gradient centrifugation and extracting DNA from it. 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 seronegative period during the first week of illness (Richards, 2012), 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 early and optimal selection of treatment. There were 16 cases among the 319 ST positive cases which were positive by n-PCR only (means among the 319 cases, 16 cases were positive only by n-PCR and in these 16 cases, all other tests were negative). Fifteen of these cases had fever of ≤1 week, indicating the early seronegative stage in ST. In such early stage of the illness, n-PCR or other molecular test could be the test of choice. Additionally, there were 5 of the n-PCR cases that were negative by M-IFA but positive for either IgM ELISA or WFT. This is very surprising, and possible reasons could be the differences in the genetic nature of the infecting isolate and warrant further research.

4.5. Degree of agreement between test and combination of tests

Individually, M-IFA and n-PCR detected 298 and 240 cases, respectively, but when combined, 319 cases were positive, 21 cases more than that of M-IFA alone ($P < 0.001$), showing the importance of molecular test in early and accurate diagnosis. Individually, the highest measure of agreement was observed for M-IFA ($\kappa = .952$), followed by n-PCR ($\kappa = .808$), ELISA ($\kappa = .785$), and WFT (OX-K) ($\kappa = .526$) (Table 5). IFA is generally not an ideal test in a rural setting as it is generally expensive (manufactured in developed countries) and need to be imported (this further increased the cost of the test; current import duties in India are approximately 35% of the kit cost). In IFA's absence, the highest diagnostic potential is seen for IgM ELISA and n-PCR combination with a sensitivity and specificity of 96.5% and 95.9%, respectively, and very good measure of agreement $\kappa = .911$. WFT + n-PCR combination had a sensitivity of 90.9% and a specificity of 93.7% ($\kappa = .835$), and WFT and IgM ELISA had the least sensitivity and specificity of 83.1% and 93.2%, respectively ($\kappa = .766$). It is clear from the results that when n-PCR is one of the tests in combination, the κ value was very good, while in its absence, it decreased. For laboratory diagnosis of ST, one must consider primarily the time course of the infection and, importantly, the initial seronegative stage and later stage of posttreatment with antirickettsial antibiotics (Richards, 2012). Antibody levels increase logarithmically from the second week of illness, reaching the peak during the early third week of illness, and serological tests should be the test of choice in this duration. However, there is no clear demarcation on these stages. Based on our results, we recommend to perform a combination of molecular and serological tests in ST diagnosis such that whole course of the infection is covered.

Recently, rapid diagnostic tests (RDTs) such as lateral flow immunochromatographic assays have become available and been found to be superior to WFT (Anitharaj et al., 2016; Stephen et al., 2016); however, these tests need to be validated locally before using them in patient care as these assays are constructed based on recombinant antigens from few of the strains of *Orientia tsutsugamushi*. Diagnostic validation of RDT against more sensitive assays such as IFA in statistical Indian population is still needed.

Availability of resources is a limiting factor in resource-limited settings, and the unavoidable shortcomings of the study were failure to simultaneously detect IgG antibodies and noninclusion of additional molecular tests such as groEL PCR which may have possibly identified more cases. Further, as it was a hospital-based cross-sectional study involving inpatients, selection bias could not be avoided. The information on prevalence of other rickettsial diseases in our population is yet to be well understood.

In conclusion, early diagnosis of ST on acute clinical samples requires a combination of serological and molecular tests, preferably IFA and n-PCR. Clinicians and microbiologists need to be aware of the diagnostic fallacies of individual tests for ST and know the utility of better/ combination assays in patient care.

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Disclosures

No authors have any conflicts of interest to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diagmicrobio.2018.01.018.

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