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SHORT COMMUNICATION

Molecular Diagnosis of Scrub Typhus: Sample and Timing Matter

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Abstract

Objective: Scrub typhus (ST) is an acute febrile illness caused by *Orientia tsutsugamushi*. Laboratory tests are needed to confirm the diagnosis when the characteristic eschar is absent. This study aimed to evaluate the performance of 47 kDa qPCR using clotted and EDTA-treated blood among patients with suspected ST

Methods: Clotted blood samples from 284 inpatients (IPs) and EDTA-treated blood samples from 194 outpatients (OPs) with suspected scrub typhus were collected from patients who were blood culture- and malaria-negative. ST IgM ELISA and 47 kDa qPCR were performed for detection of ST infections.

Results: Among the IPs (n=284) and OPs (n=194), 41% and 63% were confirmed to have ST based on the case definition, respectively; the mortality rate was 1%. The 47 kDa qPCR sensitivity of the buffy coat (65.6%) was greater compared to clotted blood (37.1%). PCR was more likely to be positive in the 1st week of the illness; IgM ELISA positivity increased after the 5th day of the illness.

Conclusion: The type of sample and the time of sample collection have a role in the diagnosis of ST. These preliminary results need to be confirmed by prospective multicentric studies.

Key words: PCR, *Orientia tsutsugamushi*, IgM ELISA, eschar, mortality

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INTRODUCTION

Scrub typhus (ST), which is caused by *Orientia tsutsugamushi* and spread by the mite (chigger). This zoonotic disease has now been described beyond the 'tsutsugamushi triangle,' with reports from South America (Chile), Europe (France), East Africa (Kenya), and Dubai [1]. ST is an acute febrile illness characterised by the presence of an eschar at the site of the mite bite [2]. The eschar is observed in 7%–97% cases of ST [3,4]. An eschar practically clinches the diagnosis because when present, an eschar has a specificity of 99% [4] and is related to the thorough-ness of the physical examination [5]. In the absence of an eschar, the diagnosis of ST requires laboratory testing because ST mimics dengue, leptospirosis, malaria, and typhoid fever [5,6]. *O. tsutsugamushi* is intrinsically resistant to the ß-lactams, fluoroquinolones, and aminoglycosides, but susceptible to doxycycline, chloram-phenicol, or azithromycin [7].

In geographic regions with a demon-strated prevalence of *O. tsutsugamushi* (endemic areas), empirical treatment to treat ST is initiated while awaiting labora-tory confirmation [5]. The methods used

for laboratory confirmation include cell culture, antigen detection, nucleic acid amplification tests (NAATS), and detection of antibodies by an immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay [ELISA] [5,6,8]. Isolation of the ST agent and IFA require expertise and infrastructure which are beyond the capability of most diagnostic centres [4,8]. Detecting IgM antibodies against the immunodominant 56 kDa antigen by ELISA is more feasible for routine diagnosis because objective results are obtained, testing can be automated [2,4,5,9], and ELISA has a similar sensitivity to IFA [9]. Conventional (cPCR), nested PCR (nPCR), and real-time PCR (qPCR) assays have been described and used for detecting *O. tsutsugamushi* 56-kDa, 47-kDa, 16 SrRNA, and GroEL gene targets [5,6,8,10]. Even though nPCR is very sensitive, there is a high risk of false-positive reactions due to contamination [11]. The qPCR has good sensitivity, provides faster results with a minimal risk of contamination, and can also be used for quantification [12]. Sensitivity ranges from 50% to 83%, whereas specificity is nearly 100% [12-14]. In this paper we have described the performance characteristics of the 47 kDa qPCR using DNA extracted from blood clots and whole blood.

MATERIALS AND METHODS

Subject recruitment

From August 2015 through December 2017, clotted blood samples from 237 inpatients (IPs) with suspected acute undifferentiated febrile illnesses (AUFIs) were collected. From August 2018 to December 2019, whole blood specimens (EDTA-treated blood samples) were collected from 194 outpatients (OPs) with AUFIs. The inclusion and exclusion criteria used for sample recruitment are shown in Table 1. Samples from study subjects were collected after obtaining informed consent. The presence or absence of eschar and response to rickettsia-specific treatment were recorded. For the IP study (blood clot PCR), subjects were recruited from the wards (inpatients). The OP group (buffy coat PCR) was recruited from individuals presenting to the adult and paediatric emergency departments (EDs) and OP clinics. The study was initiated and samples collected after obtaining ethical clearance from the Institutional Review Board (IRB [IRB min. nos. 9831 dt 07/01/2016 and 11013 dt 4/12/2017]).

ST IgM ELISA

O. tsutsugamushi anti-IgM antibodies in serum or plasma were detected using the Scrub Typhus Detect IgM ELISA System (InBios International, Inc., Seattle, WA, USA) according to the standard operating procedure (SOP) routinely followed in the Immunology Laboratory. The Immunology Laboratory is part of the ISO 15189:2012 accredited Clinical Microbiology Department. The sample with an optical density (OD) value ≥1.00 was considered positive for ST, as described previously [2]. For the IP study plasma was used for detection of ST IgM antibodies by ELISA for the IP study because serum and plasma give comparable results by PCR [15]. This finding has been further corroborated by our in-house studies (data not shown).

Real-time polymerase chain (qPCR) reaction

Each blood clot was triturated in a sterile mortar and pestle and EDTA blood was centrifuged at 1690 × g for 15 min in a refrigerated centrifuge (centrifuge 5804R; Eppendorf AG, Hamburg, Germany) to obtain the buffy coat. DNA was

TABLE 1 | Study criteria and samples used.

Criteria	Attribute	Blood clot	Buffy coat
Inclusion	Age	≥1 year	≥1 year
	Gender (M/F)	Both genders	Both genders
	Fever (≥100°F/≥37.8°C)	Yes	Yes
	Duration of fever (days)	≥5	≥3
	OP or IP	IP	OP (Emergency room)
Exclusion	Localizing signs	Yes	Yes
	Malaria	Yes	Yes
	Septicemia	Yes	Not investigated
	Enteric fever	Yes	Not investigated
Samples	ST IgM ELISA	Serum	Plasma
	47 kDa qPCR	Blood clot	Buffy coat
Subjects	Number recruited	284	194
	Eschar	63	71
	Confirmed cases	116	122

TABLE 2 | Performance characteristics of ST diagnostic parameters (IP study).

(n=284)	Eschar		IgM ELISA (serum)		47 kDa qPCR (clot)	
	Positive	Negative	Positive	Negative	Positive	Negative
ST case (n=116)	59	57	104	12	43	73
Non-ST case (n=168)	4	164	2	166	0	168
Sensitivity % (95% CI)	50.9 (41.4–60.2)		89.6 (82.6–94.5)		37.1 (28.3-46.5)	
Specificity % (95% CI)	97.6 (94.0–99.4)		98.8 (95.7–99.9)		100 (97.8–100)	
*LR+ (95% CI)	21.4 (7.9–57.2)		75.3 (18.9–300.1)		-	
*LR– (95% CI)	0.5 (0.4–0.6)		0.1 (0.1–0.2)		0.6 (0.6–0.7)	
Accuracy % (95% CI)	78.52 (73.3–83.2)		95.1 (91.9–97.3)		74.3 (68.8–79.3)	

*LR - likelihood ratio.

TABLE 3 | Performance characteristics of ST diagnostic parameters (OP study).

(n=194)	Eschar		IgM ELISA (Plasma)		47 kDa qPCR (Buffy coat)	
	Positive	Negative	Positive	Negative	Positive	Negative
ST case (n=122)	69	53	89	33	80	42
Non-ST case (n=72)	2	70	1	71	0	72
Sensitivity % (95% CI)	56.6 (47.3–65.5)		73.0 (64.2–80.6)		65.6 (56.4–73.9)	
Specificity % (95% CI)	97.2 (90.3–99.7)		98.6 (92.5–99.9)		100 (95.0–100.0)	
*LR+ (95% CI)	20.4 (5.2–80.6)		52.5 (7.5–368.9)		-	
*LR– (95% CI)	0.5 (0.4–0.6)		0.3 (0.2–0.4)		0.3 (0.3–0.4)	
Accuracy % (95% CI)	71.7 (64.8–77.9)		82.5 (76.4–87.6)		78.4 (71.9–83.9)	

*LR - likelihood ratio.

TABLE 4 | Consolidated results of performance characteristics of ST diagnostic tests.

(n=478)	Eschar		IgM ELISA (serum)		47 kDa qPCR (clot)	
	Positive	Negative	Positive	Negative	Positive	Negative
ST case (n=238)	128	110	193	45	123	115
Non-ST case (n=240)	6	234	3	237	0	240
Sensitivity % (95% CI)	53.8 (47.22–60.2)		81.1 (75.5–85.9)		51.7 (45.2–58.2)	
Specificity % (95% CI)	97.5 (94.6–99.1)		98.8 (96.4–99.7)		100 (98.5–100)	
*LR+ (95% CI)	21.5 (9.7–47.8)		64.9 (21.1–200)		-	
*LR– (95% CI)	0.47 (0.4–0.5)		0.19 (0.15–0.3)		0.48 (0.42–0.6)	
Accuracy % (95% CI)	75.7 (71.6–79.5)		89.9 (86.9–92.5)		75.94 (71.9–79.7)	

*LR - likelihood ratio.

extracted from the triturated blood clot and buffy coat using the QIAamp DNA mini-kit (QIAGEN GmbH, Hilden, Germany). Extracted DNA was stored at −20°C. RNAse P gene amplification was performed on a subset of the samples to assess the efficiency of DNA extraction [16]. The 47 kDa qPCR was performed using primers and probes described by Jiang et al. [17], which were validated by Kim et al. [12] and interpreted as described previously [16].

A composite case definition was used to identify ST cases, as described previously [2]. In brief, a patient with non-malarial fever was considered to be a ST case if either or both of the following criteria were satisfied: 1. ST-specific DNA was detected by 47 kDa qPCR; and/or 2. ST IgM ELISA was positive with defervescence within 72 h of initiating treatment with rickettsia-specific ther-apy (doxycycline or azithromycin).

Statistical analysis

All the study data, including the test results, were stored in Microsoft Excel (2016). Categorical data were expressed as frequencies and percentages. The chi-square test was used to associate the categorical variables, and the sensitivity

and specificity were calculated. All these statistics were performed using STATA I/C 13.1 (StataCorp LLC, College Station, TX, USA).

RESULTS

Amongst the 284 patients recruited in the IP study, 116 (41%) were confirmed to have ST based on the case definition, whereas 122 (63%) of 194 patients were diagnosed with ST in the OP study using the same criteria. RNAse P gene amplification performed on a subset of samples consistently had Ct values between 22 and 28 cycles, thus confirming the adequacy of DNA extraction. The performance characteristics of eschar, ST IgM ELISA, and 47 kDa qPCR, as determined against the reference standard, are as shown in Tables 2 and 3. The consolidated results of the three parameters are provided in Table 4. Representative pictures of the eschar and the amplification plot of 47 kDa are shown in Figs. 1 and 2.

DISCUSSION

In the present study we compared the performance of eschar, IgM ELISA, and qPCR with an expert-derived case definition (composite criteria) for the diagnosis of ST. In this study we did not use the 'serologic gold standard' (IgM IFA) because IgM ELISA has been reported to be equally sensitive, specific, less cumbersome, and the interpretation is objective [5].

Of the 478 patients studied, ST was diagnosed in 238 according to the case definition. A low mortality rate (1%) was observed compared to 12.2% in 2010 [18] and 4.6% in 2015 [19] from our centre. There has been a steady decline in ST-related mortality over the past decade that can be attributed to better awareness of the disease amongst the clinicians at our centre and the community [2].

As reported in the literature, the prevalence of eschar varies. In 2003 when ST re-emergence was noted for the first time in India, the eschar rate was <10% in our own
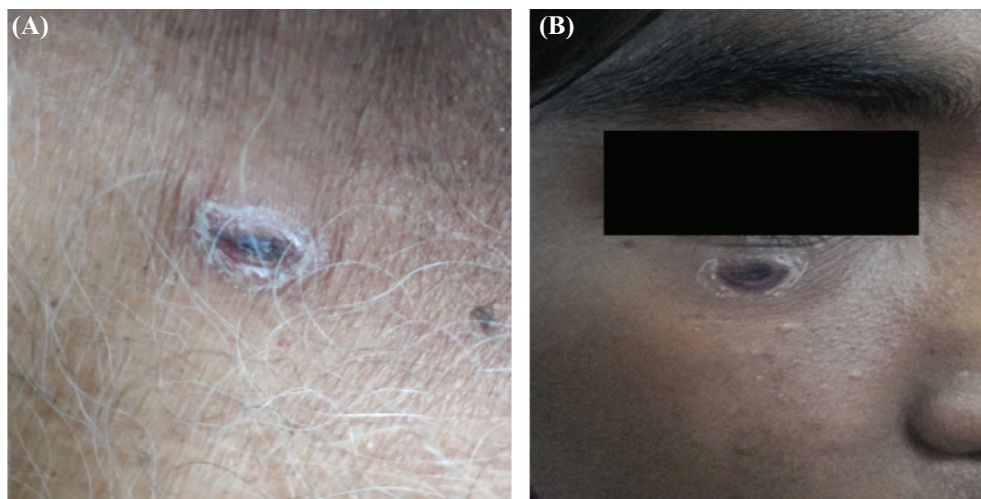
FIGURE 1 | Eschar at the nape of the neck (A), Eschar below the right eye (B).
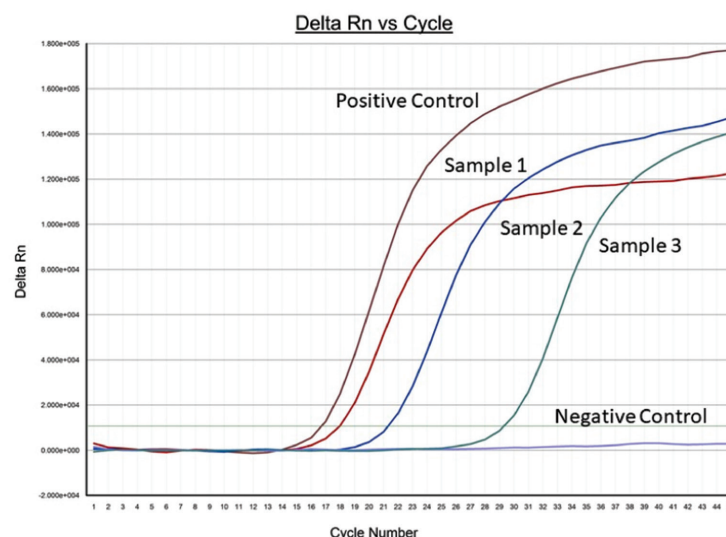
FIGURE 2 | Amplification plot: 47 kDa qPCR.

institution [20]. The eschar rate improved to 55% by 2013 [21] with increased clinician awareness and was similar to the eschar incidence (56.3%) reported in the current study.

Using the ST case definition (composite standard), the specificity of the ST IgM ELISA and 47 kDa qPCR approached 99% and reached 100%, respectively. The high specificity of ST IgM ELISA attributable to study design (clear-cut inclusion and exclusion criteria) were studied.

IgM ELISA and 47 kDa qPCR in the IPs and OPs had sensitivities of 89.6% and 73%, and 37.1% and 65.6%, respectively, for the diagnosis of ST (OD ≥1) in the current study. We attribute this variation in sensitivity to the sampling time frame (≥5 days) for the IP and OP studies (≥3 days). This finding reflects the increased likelihood that PCR is positive in the 1st week and IgM ELISA positivity increases after the 5th day of illness, as reported previously [2]. The sensitivity and specificity of IgM ELISA in the current study are comparable to previously reported studies from our centre [2,14,22,23] and elsewhere [24]. The ELISA is more objective and reproducible than IgM IFA, can be automated, and is less prone to inter- and intra-observer variation [5,25,26]. The data generated in this study reiterated the diagnostic utility of IgM ELISA using a cocktail of antigens, including the *O. tsutsugamushi* recombinant 56 kDa (r56 kDa) antigens of Karp, Kato, Gilliam, and TA716 strains [27].

The sensitivity of 47 kDa qPCR varies between 63–81% [12,27-29]. In the current study the sensitivity of 47 kDa qPCR was low with clotted blood (37.1%) collected from OPs and was higher in buffy coat samples (65.6%) collected from IPs. The variation in the two samples can be attributed to sample timing and type. Moreover, IPs had more severe infections and were likely to have a higher *O. tsutsugamushi* DNA load, as noted by Sonthayanon and co-workers [30]. It is noteworthy that the 47 kDa qPCR helped identify cases with negative serologic findings, which increased the diagnostic yield by 18% in the current study. The sensitivity of the buffy coat was closer to the lower limit of the above-mentioned studies. This finding is based on the following: the sample used by the above-mentioned studies were buffy coat or whole blood in which the yield was more compared to clotted blood, even though we had good quality DNA extracted from our samples; and the higher sensitivity in these studies were retrospective and samples were selected from confirmed cases (a four-fold rise in IgM antibodies) and non-cases by IFA. Other reasons for the low yield of qPCR in the current study could be due to genotypic variation [31] or the low *O. tsutsugamushi* copy level in the tested sample [9,32]. Additional prospective studies involving a comparison of eschars, eschar swabs, and whole blood are warranted from clinically-suspected cases of ST. Our qPCR results matched those of the prospective study conducted by Viet and co-workers [33], who observed a sensitivity 25% for 56 kDa qPCR in whole blood. Furthermore, a prospective investigation involving the diagnosis of ST in 180 febrile patients undertaken by Watthanaworawit et al.

[11] demonstrated a sensitivity of 28.6% for 47 kDa qPCR with only 2 of 7 positive blood samples. The results of the Watthanaworawit et al. [11] study were also comparable to our study, although the number of samples tested and resulting positives in our study was larger. A majority of IgM ELISA positives (83%) occurred after 5 days of illness, as previously reported [34,35]. The majority of qPCR positivity (89%) was observed before the 11th day of illness in our study group, which is also in agreement with other observations [9,30,36].

Our data suggested that the composite case definition evaluated in this study is an excellent tool for increasing the diagnostic yield of ST, which led to effective treatment. Therefore, we suggest that a sequential approach is useful for the diagnosis of ST in AUFI cases, especially those without an eschar in endemic regions. This approach includes simultaneous testing for ST by IgM ELISA and 47 kDa qPCR following malaria screening and negative blood cultures at 48 h, especially in the 1st week of illness. Further prospective multicentric studies are warranted to confirm the usefulness of this approach using buffy coat and/or eschar samples.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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