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ORIGINAL ARTICLE

Spotted fever diagnosis: Experience from a South Indian center

Elangovan D[a], Perumalla S[a], Gunasekaran K[b], Rose W[c], Verghese V P[c], Abhilash K PP[d], Prakash JAJ[a] and Dumler JS[e]

[a]Department of Clinical Microbiology, Christian Medical College, Vellore, India; [b]Department of General Medicine, Christian Medical College, Vellore, India; [c]Department of Paediatric Infectious Diseases, Christian Medical College, Vellore, India; [d]Department of Emergency Medicine, Christian Medical College, Vellore, India; [e]Department of Pathology, Uniformed Services University of Health Sciences, Bethesda, MD, USA

ABSTRACT

Spotted fever (SF) is an important treatable cause of acute febrile illness (AFI) with rash and has reemerged in India. A prospective AFI with rash study was undertaken at a South Indian hospital to correlate specific clinical findings with laboratory confirmation of spotted fever. During the study period (December 2017 to May 2019), 175 patients with fever and rash were suspected to have spotted fever. Molecular assays for scrub typhus and spotted fever (47 kDa and ompA qPCR) and serology (IgM ELISA) was performed on the 96 individuals recruited. Laboratory confirmed SF cases (ompA qPCR positive) were 21, whereas laboratory supported SF cases (ompA negative but sero-positive by SF IgM ELISA) were 27. Among the 48 spotted fever (SF) cases, 70% of had maculopapular rash, 12.5% had macular rash, purpuric/petechial rash (severe rash) was seen in 8 patients (16.7%). Presence of rash on the palms and soles was associated with a relative risk (RR) of 4.36 (95% CI: 2.67–7.10; $p < 0.001$). Our study suggests that ompA qPCR though useful for confirming the diagnosis of spotted fever is not always positive. A positive SF IgM ELISA in febrile individuals with palmo-plantar rash supports the diagnosis of spotted fever especially when other causes of febrile rash have been excluded. Multi-centric prospective studies employing the serological reference standard, IFA (immunofluorescence assay) in addition to the assays used in this study are needed to validate these findings.

KEYWORDS

Spotted fever; India; AFI; ompA; gltA; IgM ELISA; rash; palms and soles

Introduction

Spotted fever is an important treatable cause of acute febrile illness (AFI) with rash [1–3]. The illness is caused by vector-borne pathogenic species in the spotted fever group of the genus *Rickettsia* (SFGR). The initial features include fever, rash, headache, myalgia and malaise which are therefore difficult to distinguish from other causes of exanthematous fevers [4,5]. The rash could be maculopapular, petechial, purpuric or vesicular [6] and often appears 2 to 5 days or longer after the onset of fever [7–9]. Although eschars can be observed in 22–100% [7,8,10], eschars are not noted in spotted fever studies from our center [8,11]. As there are many entities in the differential diagnosis of acute fever with rash, laboratory testing is always needed for confirmation of the diagnosis of spotted fever [11,12].

Diagnosis of spotted fever can be achieved by cell culture, antigen detection, antibody detection or by nucleic acid amplification using PCR [10–12]. Of these, serological assays are the mainstay of laboratory diagnosis of spotted fever [7,11–13]. Though the indirect immune-fluorescent assay (IFA) is the preferred test, it requires expertise for performing and interpreting [7,13]. Moreover, requirement of paired samples for confirmation is not always feasible [13]. Spotted fever IgM ELISA, though not ideal [14], is used as it is inexpensive, gives an objective result, has less intra-assay and inter-assay variation and can be automated to screen large number of samples [15]. For this reason, in India, and at our center, IgM ELISA is used routinely in the appropriate clinical setting for the serological diagnosis of spotted fever [8,16–18] especially after excluding other causes of acute febrile illness with rash.

In the recent past, robust molecular assays like PCR became available for rapid diagnosis of rickettsial infections [12]. PCR assays amplifying 16SrRNA, *ompA, ompB*, and the 17-kDa lipoprotein gene targets have been used for molecular confirmation of spotted fever [11]. In conventional methods, detection of the amplicon requires gel electrophoresis, increasing turn-around time. The major disadvantage of nested and semi-nested PCR is potential for amplicon contamination as it requires a two-step process [19]. Real-time PCR shows good results, with less risk of contamination, shorter turn-around time and can be used for quantification of DNA. The limitation of PCR is that the equipment and reagents are costly [6] and requires specially trained staff for performing the assays [6,7]. Also, PCR sensitivity is directly related to rickettsemia and sample timing [12].

CONTACT Prakash JAJ prakjaj@cmcvellore.ac.in Department of Clinical Microbiology, Christian Medical College, Vellore 632004, India

Amplification of *ompA* confirms the identity of the organism as spotted fever group *Rickettsia* (SFGR) [11]. In a previous study, a *Rickettsia* genus-wide citrate synthase gene (*gltA*) was evaluated in biopsies from skin rash. Of 58 patients, 38 were found to be positive by *gltA* nPCR. While the nested PCR has good sensitivity, it is cumbersome, time consuming and prone to contamination and false-positive results in comparison to real-time PCR [11]. For detecting spotted fever DNA, skin rash biopsy (or eschar crust/swab) is considered the best specimen [12] and whole blood and/or buffy coat are commonly used as they are easily available [12,15].

In this study, we present our experience of diagnosis of spotted fever in individuals with acute febrile illness with rash.

Materials and methods

This prospective study was undertaken in the Department of Clinical Microbiology, Christian Medical College, Vellore from December 2017 to May 2019. Following Ethics Committee clearance (IRB min No. 11013 dated 4 December 2017), eligible individuals were consented into the study. The study population included individuals of either sex and any age with acute undifferentiated fever (without localizing signs) of ≤15 days' duration and rash whose samples were sent for spotted fever *Rickettsia* serology. Exclusion criteria included those with malaria, sepsis, enteric fever, drug-induced rashes, pustular rashes, viral exanthem or enanthem. Individuals with drug rash, pustular rashes, viral exanthems and enanthems were excluded by the physicians using detailed history and physical examination. Malaria was ruled out by testing three consecutive EDTA blood specimens by quantitative buffy coat (QBC venous tube, Tosoh India Pvt Ltd, Thane, India). Automated blood culture system (BacT/Alert, bioMerieux, Durham, NC, USA) was used to exclude enteric fever and septicemia (minimum one set of two bottles each). ELISA to detect NS1 Antigen, IgM and IgG antibodies (J. Mitra & Co. Pvt. Ltd., New Delhi, India) was performed to exclude dengue.

Whole EDTA-anticoagulated blood for buffy coat preparations and blood for serum were collected from all patients after informed consent. Scrub typhus (ST) ELISA to detect IgM antibodies to *Orientia tsutsugamushi* (Scrub Typhus Detect IgM ELISA System, InBios International, Inc., Seattle, WA, USA) and spotted fever (SF) ELISA for rickettsia (*Rickettsia conorii* IgM ELISA, Vircell, Granada, Spain) were performed on the serum samples. IgM antibodies were considered positive if the OD was ≥1.0 at a serum dilution of 1:100 for scrub typhus [20] and for spotted fever based on our in-house studies (Prakash JA, personal communication). While IgM testing of the initial serum sample has great limitations when applied to all AFIs largely owing to low specificity [14], our prior validation demonstrated 95% specificity when 173 non-rickettsial sera were tested (data not shown). Since the study excluded the major known causes of AFI in our region as described, and our prior work demonstrated that the prevalence of spotted fever rickettsiosis in a population that excludes the most common causes of AFI increases to at least 59% [11], we estimate that the positive predictive value of acute phase IgM under these circumstances is approximately 97%.

Scrub typhus-specific DNA was detected by amplifying the 47 kDa (*htrA*) gene by real-time PCR (qPCR) as described by Jiang et al. [21]. Spotted fever DNA was detected by a nested *gltA* (citrate synthase) PCR which has been used before in our laboratory [11]. The *ompA* qPCR primers and probe used in this study amplify a 118 bp fragment of the *ompA* gene which is specific for the spotted fever group *Rickettsia* (SFGR). The sequence of the forward primer (ompA F) sequence used was TGGTCAGGCTCTGAAGCTAAAC, the reverse primer (ompA R) sequence was AGCACCTGCCGTTGTGATATC and the TaqMan probe sequence was FAM-TAGCCGCAGTCCCTACAACACCGC-BHQ1. The analytical sensitivity and specificity of this *ompA* qPCR has been determined previously [22]. DNA extracted from buffy coat samples using the QIA amp Mini Kit (Qiagen, Valencia, CA, USA) was used as template for the PCR assays. To confirm fidelity of amplification, two *gltA* amplicons were sequenced as described before [11]. RNAse P gene amplification was performed to assess DNA extraction efficacy and for detection of PCR inhibition as previously described [23]. Where possible, skin biopsy of the rash was collected after patient gave consent.

We classified the study subjects into three groups in accordance with CDC diagnostic criteria for spotted fever rickettsiosis (https://wwwn.cdc.gov/nndss/conditions/spotted-fever-rickettsiosis/case-definition/2010/) as described below:

Clinically suspected case of spotted fever: An individual with acute undifferentiated febrile illness (AUFI) with rash was considered as a probable case of spotted fever if malaria, enteric fever, septicemia, scrub typhus, drug rash and viral exanthems and/or enanthems were excluded.

Laboratory supported case of spotted fever: Suspected case which was negative by PCR but seropositive by SF IgM ELISA, other etiologies for fever with rash excluded and with defervescence of fever within 72 hours of initiating treatment with doxycycline or azithromycin.

Laboratory confirmed case: Suspected case who was positive by ompA real-time PCR performed on

Table 1. Assay positivity and treatment response among SF cases (n = 48).

Case type	Buffy coat qPCR	Skin Biopsy qPCR	IgM ELISA	Treatment response	Number
Laboratory confirmed SF	Negative	Positive	Positive	Yes	2
(n = 21)	Positive	Positive	Positive	Yes	1
	Positive	Not done	Positive	Yes	12
	Positive	Not done	Negative	Yes	6
Probable SF	Negative	Negative	Positive	Yes	1
(n = 27)	Negative	Not done	Positive	Yes	26

Table 2. Details of spotted fever PCR positive patients with negative IgM ELISA.

Patient no.	1	2	3	4	5	6
Age (in years)	1	9	1	1	1	21
Gender	M	M	F	F	M	F
Fever duration (days)	4	12	5	6	8	10
Day rash appeared	2	2	3	5	3	2
Rash type		Maculopapular			Macular	Maculopapular
Rash on palms &soles	Yes	Yes	No	Yes	Yes	No
Pedal edema	Yes	No	No	No	No	Yes
Hepatomegaly	Yes	No	No	No	No	No
Antimicrobial agent			Doxycycline			Azithromycin

DNA extracted from EDTA blood (buffy coat) or skin biopsy.

Statistical analysis

Data was entered in Excel 2013 and the analysis was performed using STATA 15.1 (StataCorp LLC, College Station, TX, USA). Descriptive statistics were given as frequency with percentages for all the categorical variables and mean with standard deviation for the continuous variables. The data analyzed included variables such as: sex, age, duration of fever, day of appearance of the rash, severity of rash, presence of rash on the palms and soles, pedal edema and hepatomegaly. Log-binomial regression producing risk ratio (RR) with 95% CI and p-value were calculated for these to determine association with SF. For analysis, confirmed and laboratory supported cases were considered as cases.

Results

A total of 175 patient samples were sent for spotted fever serology, of these 37 did not wish to participate and 42 did not have rash. The remaining 96 of these were suspected to have spotted fever and samples were collected (EDTA anti-coagulated blood and clotted blood). In addition, in three patients' skin biopsy samples were also collected. Laboratory confirmed cases and laboratory supported spotted fever cases were 21 and 27, respectively, details are as given in Table 1. Among the 21 laboratory confirmed cases (*ompA* qPCR positives) only 8 were *gltA* nPCR positive. As other common causes of fever with rash were effectively ruled out, the laboratory supported cases (n = 27) were combined with laboratory confirmed cases (n = 21) into a group of 48 SF cases.

Among these 48 spotted fever (SF) cases, 41 (84%) were children. Male (n = 28) to female (n = 21) ratio among these was 4:3. Rash appeared by the fifth day after fever onset in 45 (92%) of the 48 cases. Maculopapular rash was observed in 34 (70.8%) patients; while six (12.5%) had macular rash, purpuric or petechial rash (severe rash) was seen in 8 patients (16.7%). Rash on palms and soles was observed in 35 (71.4%), pedal edema in 19 (39.6%) and hepatomegaly in one (2.1%) case. Among the 48 patients, 43 received doxycycline and 5 received azithromycin, and all demonstrated defervescence of fever within 72 hours of initiation of therapy. *Rickettsia*-specific therapy (doxycycline or azithromycin) was initiated in 44 (90%) of the 48 patients before samples were sent for spotted fever diagnostic assays. None of our spotted fever cases had eschar and there were no fatalities.

There were six false negatives by SF IgM ELISA who were positive by *ompA* qPCR. They all promptly responded to rickettsia-specific treatment; the salient details of these six individuals are given in Table 2. SF IgM ELISA results were false positive in nine patients. Of these, five patients had scrub typhus based on the presence of *O. tsutsugamushi* DNA (*htrA*) by 47 kDa qPCR, and one each had Hepatitis A virus infection, Stevens-Johnson Syndrome, impetigo (streptococcal) and Reye Syndrome. The RNaseP qPCR Ct values had a mean value of 24.2 ± 1.5 (Range 21.2 to 27.3) for all the buffy coat samples tested by qPCR.

As the prevalence of SF was 50%, risk ratios (RR) were estimated using log-binomial regression as logistic regression analysis overestimates the results. Univariate analysis was performed to assess relative risk in predicting diagnosis of spotted fever (SF). Age, sex, duration of fever, relationship of rash to onset of fever, severity of rash, presence of rash on the palms and soles, pedal edema and hepatomegaly were the

Table 3. Risk ratio analysis.

	SF case		Univariate analysis		Multivariate analysis	
Variables	Yes (n = 48)	No (n = 48)	RR (95% CI)	p-Value	RR (95% CI)	p-Value
Gender						
Females	21 (43.8%)	20 (41.7%)	1.00			
Males	27 (56.3%)	28 (58.3%)	0.96 (0.64–1.43)	0.836		
Age (Years)	11.8 ± 17.5	19.1 ± 20.3	0.97 (0.97–1.00)	0.069		
Age group						
Children	40 (83.3%)	32 (66.7%)	1.67 (0.91–3.04)	0.096		
Adults	8 (16.7%)	16 (33.3%)	1.00			
Fever duration (days)	8.0 ± 2.8	8.3 ± 4.7	0.99 (0.94–1.05)	0.787		
Rash appearance#	3.3 ± 17	3.4 ± 2.9	0.99 (0.91–1.08)	0.880		
Type of rash						
Severe*	8 (16.7%)	10 (20.8%)	0.87 (0.50–1.52)	0.616		
Non-severe**	40 (83.3%)	38 (79.2%)	1.00			
Palmo-plantar rash	35 (72.9%)	1 (2.1%)	4.49 (2.76–7.28)	<0.001	4.36	<0.001
Yes	13 (27.1%)	47 (97.9%)	1.00		(2.67–7.10)	
No						
Pedal edema						
Yes	19 (39.6%)	5 (10.6%)	1.94 (1.34–2.74)	<0.001	1.12	0.030
No	29 (60.4%)	42 (89.4%)	1.00		(1.01–1.25)	
Hepatomegaly						
Yes	1 (2.1%)	2 (4.2%)	0.78 (0.45–1.34)	0.364		
No	47 (97.9%)	46 (95.8%)	1.00	1.00		

The day rash appeared.
*Severe rash: Purpuric and petechial rash; **Non-severe rash: Macular and maculopapular.

parameters evaluated (Table 3). Pedal edema and rash on the palms and soles showed a significant RR by univariate analysis, the same was confirmed by multivariate analysis (Table 3).

The two *gltA* amplicons sequenced have been submitted to the GenBank database (*http://www.ncbi.nlm.nih.gov/Genbank/*) and accession numbers (MN399865 and MN480240). The *gltA* sequence, MN 399865 demonstrated a 100% homology with our previously reported uncultured *Rickettsia sp*. Clone cmc 08 (GQ260637), 99.4% with *Rickettsia sp.Tenjiku01 (*LC089864) and *Rickettsia raoulti (*KR131756) and 99.1% with *Rickettsia parkeri*(CP040325). The other sequence (MN480240) showed a 99.4% homology with uncultured *Rickettsia sp*. Clone cmc 08 (GQ260637), 98.8% with *Rickettsia sp.Tenjiku01 (*LC089864) and *Rickettsia raoulti (*KR131756) and 98.5% with *Rickettsia parkeri*.

Discussion

Spotted fever is a common treatable cause of fever with rash [1,3] with different types of rash being observed [6–8]. Failure to treat appropriately due to lack of clinical suspicion or non-availability of diagnostic assays leads to increased morbidity and increase in hospital stay [6,24].

In this prospective study, 96 patients with fever and rash were recruited and 48 were considered as spotted fever cases, a majority (84%) of them being children. Similar findings have been reported in the literature [8,16,25,26], though Mane et al. reported a high prevalence in Gorakhpur among those who were 15–25 years old [17]. Our SF cases had no eschar and no mortality as reported in previous studies [8,9,11]. The lack of mortality is potentially due to early initiation of empiric rickettsia-specific treatment as there is a high prevalence of scrub typhus in our setting [1].

In a majority (≈90%), a macular or maculopapular rash appeared by the fifth day as reported in the literature [7,27]. Most of the cases of spotted fever occurred in cooler months as reported earlier [24]. The increase in spotted fever in cooler months is likely to be related to increased tick activity as described by Latha *et al*. in 2004 [28]. None of our cases had eschars; similar findings have been reported previously from India [8,9,11,16]. Our data suggest a strong association of rash on the palms and soles, in an individual with a febrile rash, with a diagnosis of spotted fever. These findings are similar to that reported earlier from our center [8,29] and by others [30–32].

The *ompA* qPCR provided laboratory confirmation of 21 cases of spotted fever. The combination of SF IgM ELISA, exclusion of other common forms of acute febrile illness, and defervescence of fever to treatment in individuals with febrile rashes detected 27 more cases. This observation reiterates that a combination of tests, clinical response to therapy and excluding out diseases which mimic spotted fever to increase the prevalence in the study population provides a more accurate diagnostic yield. This has been observed by Baughman and others with pertussis [33]. The *ompA* qPCR increased the diagnostic yield as it picked up six cases negative by SF IgM ELISA. Nevertheless, whole blood and buffy coat blood are not as good specimens as is skin biopsy of the rash for detection of SFGR DNA [7,10,12,15]. PCR inhibitors were ruled out as RNAse P was adequately amplified by qPCR (Ct values 24.2 ± 1.5).

IgM positivity alone is not proof of acute spotted fever. This is because SF IgM (by ELISA or IFA) may be falsely positive in other differentials for AUFI like dengue, malaria, typhoid, Q fever and scrub typhus [18,34,35]. To overcome this, we increased the likely prevalence of spotted fever through both clinical and laboratory tests by excluding other common causes of febrile illness in our region, and applied case definitions in part based on the CDC criteria (https://wwwn.cdc.gov/nndss/conditions/spotted-fever-rickettsiosis/case-definition/2010/) as described in the Methods section. As expected, we observed nine false positives for SF IgM ELISA in our study, five of which were due to scrub typhus. This is because serological assays including the reference assay, the IFA, do exhibit cross reactions [4,13].

Among the six SF IgM false negatives, four were infants who had durations of illness less than 8 days. The fifth was a pregnant woman (22 weeks) with fever for 10 days. The sixth false negative was a nine-year-old child with fever for 12 days and rash for 10 days and was from the same area from where we had reported '*Candidatus* Rickettsia kellyi' [36]. All these patients could have IgM antibodies against an infecting rickettsial species which binds inefficiently to *R. conorii* antigen (ATCC VR-141) used in the ELISA performed in this study. This is supported by the isolation of a new rickettsial species, named *Candidatus* Rickettsia indicaTenjiku01 [37] in addition to our *ompA* sequence findings regarding *Candidatus* Rickettsia kellyi [11]. The other possibility is that there could be a delayed immune response with undetectable antibodies within the first 10–14 days [7,10,12]. In this study, strict application of inclusion and exclusion criteria, data provided by the molecular and serological assays and also considering the response to treatment improved the limitations of sero-diagnostic IgM tests, and thus, the diagnostic yield. This is supported by our experience with scrub typhus [20] and is supported by the observations of Robinson *et al.* for spotted fever [15].

We justify the use of *ompA* qPCR, as molecular amplification of *ompA* gene is confirmatory for SFGR [13], whereas the *gltA*, 17 kDa gene and *ompB* gene are also found in the TGR (typhus group rickettsia). Therefore, sequence analysis of *gltA*, 17 kDa and *ompB* amplicons is needed to confirm the presence of SFGR [6,7,10,12,15,38]. The primers and probes used for amplifying *ompA* in our study were obtained from consensus *ompA* sequence derived by alignment of 23 SFGR *ompA* gene sequences their analytical sensitivity and specificity for qPCR were previously validated [22]. This assay has also been found very useful in clinical settings in Nicaragua [39] and Malaysia [40]. In this study, we observed a higher detection rate with *ompA* qPCR as compared with the *gltA* nPCR. This is similar to the observations noted by Kato *et al.* using whole blood [41] and for formalin fixed paraffin embedded (FFPE) specimens by Denison AM *et al.* [42]. Znazen and coworkers compared rickettsial qPCR and nPCR in whole blood and skin biopsy, and their results for whole blood were less than 10% [43].

Sequencing performed on two whole blood amplicons for the *gltA* gene followed by BLAST analysis provided confirmatory evidence for specificity (fidelity) of target amplification. The two *gltA* amplicons sequenced (MN 399865 and MN 480240) showed an excellent homology with spotted fever group rickettsia. Amplification and sequencing of other spotted fever-specific genes (17 kDa, *ompA* and *ompB*) will provide information regarding the phylogeny of the circulating SFGR in our area. This is of importance as novel rickettsia like '*Candidatus* Rickettsia kellyi' [36] and *Candidatus* Rickettsia indica Tenjiku01 [37] whereas in North India, there is evidence of *R. conorii* [9,30].

The strength of this study is its prospective nature, robust case recruitment methods, application of an IgM ELISA on prevalence-optimized study cohort and the use of qPCR for detection of spotted fever in all suspected cases. The study is limited by the fact that it provides data from a single center, did not use skin biopsy of rash (best specimen) for PCR assays in all patients, and did not examine the potential for SF in those lacking a rash. In addition, paired sera (acute and convalescent) were not tested by the current standard IFA for rising titers or seroconversion.

In conclusion, exclusion of other causes of febrile rash and presence of a palmo-plantar rash is strongly suggestive of spotted fever and an indication to treat it as such. The *ompA* qPCR is useful for confirming the diagnosis of spotted fever. In resource limited settings, a positive SF IgM ELISA in the appropriate clinical setting with other etiologies excluded is strongly supportive of a diagnosis of spotted fever. Further, prospective studies using IFA and paired sera (acute and convalescent) are needed to confirm these findings.

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Disclosure statement

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