



State of the art of diagnosis of rickettsial diseases: the use of blood specimens for diagnosis of scrub typhus, spotted fever group rickettsiosis, and murine typhus

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Purpose of review

With improved malaria control, acute undifferentiated febrile illness studies in tropical regions reveal a startling proportion of rickettsial illnesses, especially scrub typhus, murine typhus, and spotted fever group rickettsioses. Laboratory diagnosis of these infections evolved little over the past 40 years, but combinations of technologies like PCR and loop-mediated isothermal amplification, with refined rapid diagnosis tests and/or ELISA, are promising for guidance for early antirickettsial treatment.

Recent findings

The long-term reliance on serological tests – useful only late in rickettsial infections – has led to underdiagnosis, inappropriate therapies, and undocumented morbidity and mortality. Recent approaches integrate nucleic acid amplification and recombinant protein-based serological tests for diagnosing scrub typhus. Optimized using Bayesian latent class analyses, this strategy increases diagnostic confidence and enables early accurate diagnosis and treatment – a model to follow for lagging progress in murine typhus and spotted fever.

Summary

A laboratory diagnostic paradigm shift in rickettsial infections is evolving, with replacement of indirect immunofluorescence assay by the more objective ELISA coupled with nucleic acid amplification assays to expand the diagnostic window toward early infection intervals. This approach supports targeted antirickettsial therapy, reduces morbidity and mortality, and provides a robust evidence base for further development of diagnostics and vaccines.

Keywords

ELISA, indirect immunofluorescent assay, murine typhus, *Orientia tsutsugamushi*, PCR, rickettsia, scrub typhus, spotted fever

INTRODUCTION

The global decline of malaria revealed an array of acute undifferentiated febrile illnesses (UFI) that exact a high toll on human health [1]. Recent large-scale studies of UFI in (sub)tropical regions reveal that rickettsial diseases, predominantly scrub typhus and murine typhus, are among the leading causes of treatable UFI [2–4,5^{••},6–12]. Rickettsial illnesses are often misdiagnosed as malaria, dengue, or typhoid, and are important preventable causes of morbidity and mortality [2–4,5^{••},6,13,14]. Rickettsial infections affect the vasculature to present with nonspecific signs and symptoms rendering early clinical diagnosis difficult [15]. The dissemination dynamics of *Rickettsia* and *Orientia* with their

early limited bacteremic phase and subsequent appearance of antibodies have hindered the development of effective diagnostic tools for targeted

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KEY POINTS

- Scrub typhus, murine typhus, and to an uncertain extent spotted fever rickettsiosis are important treatable causes of UFI in the tropics and among travelers.
- ‘Days of fever prior to admission’ informs the disease phase of a rickettsial infection – bacteremia or early immune response – that prompts for genus-broad real-time PCR followed by species-specific confirmatory PCR and a serological test.
- Eschars or eschar crust – if present and accessible – represents an excellent noninvasive sample specimen for NAA, allowing for diagnosis even post initiation of treatment.
- Laboratory confirmation of murine typhus and spotted fever rickettsiosis relies on antibody tests; although PCR can provide helpful evidence, NAA in the diagnosis of SFGR and TGR is limited.
- Rickettsial infections in the tropics and in returning travelers are potentially severe, but easily treatable if diagnosed, underscoring the importance of clinical awareness and availability of affordable, accurate, point-of-care RDTs.

early antirickettsial therapy. Especially in disease endemic areas, the occurrence of high background antibody titers poses an additional challenge to the already difficult serodiagnosis [16,17^{***},18,19]. More hurdles involve translating promising technologies with high analytical sensitivity and specificity into clinically useful tests [2,20^{*}]. Here, we describe recent advances and major knowledge gaps in diagnosing rickettsial diseases, focusing on blood specimen-based tests conducted at the time of acute illness to inform targeted treatment.

SCRUB TYPHUS AND RICKETTSIOSES OVERVIEW

Scrub typhus is arguably the world’s most important rickettsial illness in terms of disease burden and is a leading cause of treatable UFI in Asia and Pacific regions where it accounts for up to 20% of febrile hospital admissions in endemic regions [2,4,5^{***},6,13]. Recent evidence of *Orientia* spp. found in Africa, Europe, and South America indicates a wide genetic diversity and geographic distribution [2]. Scrub typhus, caused by *Orientia tsutsugamushi* and transmitted by *Leptotrombidium* mites, presents with ‘flu-like’ symptoms, and often with an eschar and/or a macular/maculopapular rash. Although effectively treated with tetracyclines, macrolides, and chloramphenicol, delayed treatment responses and severe disease with case fatality rates reaching

12–13% in northern Thailand and southern India are documented. Scrub typhus remains severely underrecognized, mainly because of diagnostic difficulties and lack of awareness.

The genus *Rickettsia* is divided based on antigenic and genomic distinctions and has an enlarging taxonomy [21]. The major pathogens are globally distributed and classified primarily within spotted fever and typhus group clusters [21,22]. Among tropical and travel-related infections, murine (fleaborne) typhus, caused by *Rickettsia typhi* in the typhus group rickettsia (TGR), is a common cause of UFI in tropical areas and travelers, especially in southeast Asia [3,21,23]. The expanding spotted fever group rickettsiae (SFGR) are less well studied globally. The GeoSentinel Network describes the highest rate in travelers returning from sub-Saharan Africa [7], but seroepidemiologic data and clinical studies show high prevalences of SFGR in the Americas, Mediterranean basin, north and south of Africa, Australia, and increasingly in Asia [13,21,24–26]. Major diagnostic challenges for SFGR are the close genetic relatedness and serological crossreactivity [27]. These rickettsioses also present with ‘flu-like’ symptoms, sometimes with an eschar and/or a macular/maculopapular rash, further complicating diagnosis [28].

DIAGNOSTIC ASPECTS FOR SCRUB TYPHUS, MURINE TYPHUS, AND SPOTTED FEVER RICKETTSIOSES

Major modalities for diagnosing rickettsial illnesses include culture, nucleic acid amplification (NAA), and serology; the latter includes rapid diagnostic tests (RDTs), indirect immunofluorescence assays (IFAs), and ELISA. Antigen detection in skin, eschar, or tissue biopsies can be advantageous during the acute phase of infection, and culture is useful for definitive identification and characterization, but either invasive sampling or long incubation times and biosafety aspects render these suboptimal for acute setting diagnosis.

SCRUB TYPHUS NUCLEIC ACID AMPLIFICATION TESTS

In 1990, PCR was first shown useful for detecting *O. tsutsugamushi* in clinical specimens. Nucleic acid detection is accurate in the early phase of infection up to 10 days of fever, after which serology becomes better at diagnosing scrub typhus. Common target genes include the *htrA* (47-kDa periplasmic serine protease), 56-kDa type-specific antigen, *rrs* (16S rRNA), and *groEL* (heat shock protein Hsp60). The 56-kDa type-specific antigen gene is specific to

Orientia spp. only and PCR positivity and/or product sequencing provides strong evidence for the presence of pathogen DNA [29–32].

Real-time PCR prevails for diagnosis of scrub typhus, but high costs and training limit its use in rural areas. PCR assays are only as good as the samples used and depend on bacterial load and time point of disease. Common samples are whole blood, buffy coat, and eschars. Samples from eschar biopsies or noninvasive eschar crust are excellent for PCR, but only in areas of high eschar rates (e.g., Korea or China report >95%). Loop-mediated isothermal amplification (LAMP) assays are easy to use, need no thermocycler, provide a simple readable endpoint, and have comparable diagnostic accuracies to PCR, but are not widely used. Combined algorithms incorporating a NAA assay with an antibody-based test should be used as they expand the interval for successful laboratory diagnosis in the acute setting [33,34].

Scrub typhus rapid diagnostic tests

The availability of affordable and accurate point-of-care RDTs has improved directed treatment, and their widespread use enhances the awareness of scrub typhus. Comparisons of RDTs demonstrate improved diagnostic accuracy when using IgM over total antibody. Anti-*O. tsutsugamushi* IgG can persist leading to high RDT false-positive rates in endemic areas, for which assay adjustments might be required. Currently available RDTs are immunochromatographic or semiquantitative dot-blot assays, increasingly incorporating recombinant antigens, allowing greater standardization, and simple readout for point-of-care testing in resource-constrained settings [16,35–38].

Scrub typhus serology by indirect immunofluorescence assays

The IFA has been the mainstay in scrub typhus diagnostics for decades. However, the lack of standardization, variable cutoff titers for endemic regions, requirement for paired sera, high cost, and subjective endpoints are causes for concern [39]. The rigorous use of at least four-fold antibody titer rise in paired sera improves confidence, but confounding factors such as preexisting antibodies and cross-reactivity remain. A combination of diagnostic modalities were incorporated into the scrub typhus infection criteria (STIC), as a composite endpoint for diagnostic comparisons; STIC was considered positive if either *O. tsutsugamushi* was isolated, at least two of three PCR assays were positive, and an admission IFA IgM titer was at least 12 800, or at least

four-fold rise in convalescent IFA IgM titer was present [33]. STIC became the standard, but evaluations of new tests against a flawed gold standard inexorably lead to suboptimal biased results. Bayesian latent class modeling overcomes these difficulties, as it estimates accuracies of diagnostic tests using the true disease status of each patient (infected or noninfected), does not require a gold standard, does not assume that any diagnostic test or combination is perfect, and provides unbiased sensitivity and specificity estimates [17^{***}]. Using this analytical tool, the initial STIC recommendations improved to using a single admission IgM IFA titer of at least 3200 and/or a four-fold rise to at least 3200 in paired samples. This corrected for false positive rates associated with low-rising IFA titers and significantly increased sensitivity and specificity of the modified STIC [18].

Scrub typhus serology by ELISAs

Improved anti-*Orientia* IgM and IgG-based RDTs and ELISAs are replacing subjective IFAs. Increasingly, new assays use *O. tsutsugamushi* recombinant proteins to detect specific antibodies and have become less expensive, with improved sensitivity, specificity, and reproducibility [35,36,38,40]. ELISA offers advantages over IFA in simplicity, standardization, objectivity, and throughput. However, establishing a validated diagnostic cutoff is often overlooked, especially in endemic areas. A recent evaluation of ELISA found a strong association between optical density (OD) values and IFA titers. This enabled the determination of an ELISA optical density positive cutoff corresponding to a single IFA titer of at least 1600 with 93% sensitivity and 91% specificity [35], and is congruent with the improved composite indicators of STIC.

MURINE TYPHUS AND SPOTTED FEVER GROUP NUCLEIC ACID AMPLIFICATION TESTS

The major NAA methods for SFGR and TGR include LAMP and PCR; for PCR, a large array of gene targets are published, but none is substantially more effective than others [41]. Frequently used genes include 16S rRNA (*rrs*), citrate synthase (*gltA*), 17-kDa lipoprotein, and other conserved genes [41,42]. ‘Diagnosis-to-treat’ approaches incorporate *Rickettsia* genus-specific real-time PCRs [24,43^{***},44–46]. However, unique gene regions can be targeted for species and subspecies-level identification [21,41], or broad-range PCR amplicons can be sequenced [47], as real-time PCR target sequences of 75–150 nucleotides provide only limited taxonomic information.

Although simple and field applicable, SFGR and TGR LAMP assays are not well studied [48,49]; for murine typhus, low diagnostic LAMP accuracy is attributed to low *R. typhi* bacterial loads [48].

Frequent sample types include whole blood and buffy coat, and as with scrub typhus, skin or eschar biopsies/crusts or swabs are excellent for PCR, if available [21,50]. Although, real-time PCR provides reduced contamination, quantitation, and multiplex potential for species identification, or high-throughput analyses for epidemiologic investigations, conventional PCR methods, especially nested PCRs, are often used because of good diagnostic sensitivity and the potential for amplicon sequencing. In general, analytical sensitivity ranges from low for conventional PCR, to moderate/good (1000–10 000 genome equivalents/ml blood DNA) for nested PCR, and to good (<100–5000 genome equivalents/ml of blood DNA) for real-time PCR. Unfortunately, bacterial loads less than 100/ml blood (equivalent to 0.1 genome copies/ μ l reaction mixture) and poor DNA yield severely challenge analytical sensitivity limits and hinder NAA applicability [45,46,51,52].

High-quality clinical PCR evaluations are limited by small patient numbers, lack of prospective design, poorly controlled specificity, and a wide variety of techniques, targets, analytical approaches,

and uncertain gold standards. Among published PCR methods since 2013 with more than 10 samples compared with serological standards [43[■],44,53,54], clinical sensitivity varied from good (>75%) to very poor (<5%), with a median of 18% (interquartile range 4–30) (Table 1). For pan-*Rickettsia* PCRs, blood DNA median sensitivity was 18% (6–27%), for SFGR PCR was 42% (6–69%), and for TGR was 3% (1–18%). Despite real-time PCR's appeal, there are insufficient clinical data to conclude that these assays are superior to nested or conventional PCR for diagnosis of human rickettsioses. Although data are limited, the use of skin biopsy or eschar samples improves sensitivity for pan-*Rickettsia* (43 vs. 18%) and SFGR assays (67 vs. 42%), but not for TGR (6 vs. 3%). Real-time PCR modestly enhances clinical sensitivity vs. nested PCR among a cohort of 223 human blood and tissue samples examined for rickettsial infection (18 vs. 16%) [46]. Additional support for use of skin and real-time PCR comes from guinea pig SFGR model studies: median sensitivity of skin vs. blood detection was 31 vs. 3%; 44% for real-time PCR, 7% for nested PCR, and 3% for conventional PCR [55[■]].

Current methods for SFGR and TGR diagnosis are limited when using whole blood. This could be addressed by using skin rash or eschar biopsies, which are not always present in all patients.

Table 1. Median clinical sensitivity of PCR methods for detection of spotted fever group and typhus group rickettsia in blood and skin/eschar biopsy samples^a

Sample	<i>Rickettsia</i>	Method	Percentage clinical sensitivity		
			Number of assays	Median (IQR)	References
All	PanRick	All	145	23 (15–34)	[43 [■] ,44,54]
	SFGR		331	48 (34–65)	[53,54]
	TGR		257	5 (3–7)	[43 [■] ,44]
Skin	All	All	233	43 (7–55)	[43 [■] ,54]
	SFGR		101	67 (55–79)	
	TGR		88	6 (5–6)	
Blood	All	All	331	18 (4–30)	[43 [■] ,44]
	PanRick		101	18 (12–23)	
	SFGR		230	42 (24–56)	
	TGR		169	3 (2–10)	
All	PanRick	Real-time PCR	525	7 (4–23)	[43 [■] ,44]
	SFGR	Real-time PCR	123	23 (14–33)	
	TGR	Real-time PCR	257	5 (3–7)	
	SFGR	Nested PCR	29	31 (31–31)	[53]
	SFGR	Conventional PCR	179	69 (61–80)	[53,54]

^aDerived from studies for which serologic and PCR results on more than 10 patients were reported since 2013 identified using search terms 'rickettsia', 'spotted fever', 'typhus' and 'PCR', 'real-time PCR', 'nested-PCR', 'qPCR', 'quantitative PCR'. PanRick – assays that target the genus *Rickettsia*; SFGR – assays that target spotted fever group rickettsiae; TGR – assays that target typhus group rickettsiae. Number of assays column includes total assays reported, including some on the same samples but different approaches or targets. IQR, interquartile range.

Diagnostic improvements could include bacterial enrichment, high blood volume use, or multicopy gene targets to overcome low rickettsial loads. Thus, the PCR target itself is not the major limiting factor for increasing clinical sensitivity.

SERODIAGNOSIS OF TYPHUS AND SPOTTED FEVER GROUP RICKETTSIOSES

Serodiagnosis remains the gold standard for SFGR and TGR infections using seroconversion and four-fold antibody titer increases [20^a,41]. Specificity, where examined, tends to be good to excellent, with the potential exception of IgM assays [20^a,56]. Major disadvantages include poor sensitivity during acute infection (antibodies are often not detectable within the first 10–14 days), the indirect nature of diagnostic evidence (detection of host responses), and crossreactions with other *Rickettsia* [13,20^a,57]. Unlike with scrub typhus, development of RDTs lags for SFGR and TGR. The preferred method remains IFA despite requirement of experience for accuracy and precision [20^a,58]. ELISA and related protein immunoblot and immunochromatographic methods are described. Although some manufacturers provide data documenting IFA comparisons, there is a paucity of studies that evaluate diagnostic methods on well characterized patient samples. The use of insufficiently validated ELISAs is associated with reduced studies of confirmed SFG rickettsioses in the United States [59].

Sensitivity and specificity of serological assays for SFGR and TGR are shown in Table 2

[16,35,36,67,68,70–73]. Important limitations include the use of single samples for diagnosis and assumptions about cause based on serologic results. The latter is particularly relevant as all SFGR crossreact to some extent, as they do also with TGR, and as titers to individual species can vary considerably among poorly standardized methods such as IFA [13,20^a,57]. Most assays utilize antigens derived from *Rickettsia rickettsii* or *Rickettsia conorii*, but a positive result simply indicates a likely *Rickettsia* infection, and to a lesser extent a SFG *Rickettsia* infection. If several distinct SFGR antigens are used and titers differ by more than four-fold, the higher titer does not identify the etiologic agent. Although this could be in part resolved by crossabsorption studies, this is not a feasible approach for most clinical laboratories, is not rapid, and often does not resolve the specific cause [57,74]. For diagnostic purposes, the results are unlikely to be useful during the acute stage even if positive as the background seropositive rate in many tropical regions is either high or undefined [18,19,75]. Thus, reliance on single samples is discouraged and further confounded by a lack of specificity for IgM testing.

CONCLUSION

Rickettsial infections require early diagnosis and treatment to prevent severe outcomes, but this is rarely achieved using serology. For scrub typhus, combining NAA and IgM RDTs or ELISAs improves diagnostic accuracy and allows earlier detection. For SFGR and TGR infections, limited comparative

Table 2. Sensitivity and specificity of serological tests for confirmation of scrub typhus, spotted fever rickettsiosis, and murine typhus

Disease	Serological assay	Sensitivity (%)	Specificity (%)	References
Scrub typhus	IFA IgG	91	96	[60]
	IFA IgM	70–87	84–100	[16,17 ^{aa} ,60]
	ELISA IgG	80–97	89–98	[60–63]
	ELISA IgM	84–100	73–99	[60,64]
	ImmChrom IgG RDT	86–95	96–100	[38,60,65]
	ImmChrom IgM RDT	82–94	86–100	[35,38,40,60,65]
	Dot EIA	60–100	94–99	[36,60,66]
Spotted fever rickettsiosis	IFA IgG	85–100	99–100	[67–69]
	IFA IgM	83–85	100	[68,69]
	ELISA IgG	83	87	[68,70]
	ELISA IgM	98	94 ^a	[68]
Murine typhus	IFA IgG	≥83	≥93	[67]
	IFA IgM	53–85	99	

^aIncreasing data suggest lower specificity [56,59,68].

IFA, immunofluorescence assay; ImmChrom, immunochromatographic; RDT, rapid diagnostic test.

studies, restricted RDT availability, and poor evidence for IgM-based testing make NAA tests attractive. To reliably guide clinical decisions, NAA tests for *Rickettsia* require considerable improvement to resolve challenges of genus-wide detection and methods improvement to overcome low-level rickettsemia. Prospective clinical studies in endemic areas are a critical test for the next generation of highly sensitive diagnostics for rickettsioses.

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Conflicts of interest

There are no conflicts of interest.

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