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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Abstract

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 of rapid diagnostic tests and/or ELISA are promising for guidance for early anti-rickettsial treatment.

Recent findings: The longterm reliance on serological tests - useful only late in rickettsial infections – have led to under-diagnosis, 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 IFA by the more objective ELISA coupled with nucleic acid amplification assays to expand the diagnostic window towards early infection intervals. This approach supports targeted anti-rickettsial therapy, reduces morbidity and mortality, and provides a robust evidence-base for further development of diagnostics and vaccines.

Keywords: *Orientia tsutsugamushi*, *Rickettsia*, scrub typhus, murine typhus, spotted fever, indirect immunofluorescent assay, ELISA , PCR

Introduction

The global decline of malaria revealed an array of acute undifferentiated febrile illnesses (UFI) that extract 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 (2-12). Rickettsial illnesses are often misdiagnosed as malaria, dengue or typhoid, and are important preventable causes of morbidity and mortality (2-6, 13, 14). Rickettsial infections affect the vasculature to present with non-specific 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 early anti-rickettsial therapy. Especially in disease-endemic areas, the occurrence of high background antibody titers poses an additional challenge to the already difficult serodiagnosis (16-19). 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, 4-6, 13). Recent evidence of *Orientia* spp. found in Africa, Europe and South America indicate a potentially wider 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 under-recognized, mainly due to 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 (flea-borne) 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 cross-reactivity (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 approaches suboptimal for acute setting diagnosis.

Scrub typhus nucleic acid amplification (NAA) 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* (47kDa antigen), 56kDa type-specific antigen (TSA), *rrs* (16S rRNA) and *groEL* (heat shock protein Hsp60). The 56kDa TSA gene is specific to *Orientia* spp. only and PCR-positivity and/or product sequencing provide 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 non-invasive 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 (RDTs). 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 semi-quantitative 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 (IFAs). 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 the subjective endpoints are causes for concern (39). The rigorous use of ≥4-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 a) *O. tsutsugamushi* was isolated, b) at least two of three PCR assays were positive, and c) an admission IFA IgM titer ≥12,800, or d) ≥4-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 Modelling (LCM) overcomes these difficulties, as it estimates accuracies of diagnostic tests using the true disease status of each patient (infected or non-infected), 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 at ≥3,200 and/or a 4-fold rise to ≥3,200 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 enzyme-linked immunosorbent assays (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 OD-values and IFA titers. This enabled the determination of an ELISA OD positive cutoff corresponding to a single IFA titer of ≥1,600 with a 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 are 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). (22, 43)“Diagnosis-to-treat” approaches incorporate *Rickettsia* genus-specific real-time PCRs (24, 43-46). 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 due to good diagnostic sensitivity and the potential for amplicon sequencing. In general, analytical sensitivity ranges from low for conventional PCR, to moderate/good (1000 to 10,000 genome equivalents/mL blood DNA) for nested PCR, and to good (<100 to 5000 genome equivalents/mL of blood DNA) for real-time PCR. Unfortunately, bacterial loads <100/mL blood (equivalent to 0.1 genome copies/µL 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 > 10 samples compared to serological standards (43, 44, 53, 54), clinical sensitivity varied from good (>75%) to very poor (<5%), with a median of 18% (IQR 4-30) (Table 1). For pan-*Rickettsia*

PCRs, blood DNA median sensitivity was 18% (6-27%), for SFGR PCR, 42% (6-69%), and for TGR, 3% (1-18%). Despite real-time PCR's appeal, there is 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. Diagnostic improvements could include bacterial enrichment, high blood volume use, or multi-copy 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, 41). Specificity, where examined, tends to be good to excellent, with the potential exception of IgM assays (20, 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 cross-reactions with other *Rickettsia* (13, 20, 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, 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 reports of confirmed SFG rickettsioses in the USA (59).

Sensitivity and specificity of serological assays for SFGR and TGR is shown in Table 2 (16, 35, 36, 60-65). Important limitations include the use of single samples for diagnosis and assumptions about etiology based on serologic results. The latter is particularly relevant since all SFGR cross-react to some extent, as they do also with TGR, and since titers to individual species can vary considerably among poorly standardized methods such as IFA (13, 20, 57). Most assays utilize antigens derived from *R. rickettsii* or *R. 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. While this could be in part resolved by cross-absorption studies, this is not a feasible approach for most clinical laboratories, is not rapid, and often does not resolve the specific etiology (57, 66). For diagnostic purposes, the results are unlikely to be useful during the acute stage even if positive since the background seropositive rate in many tropical regions is either high or undefined (18, 19, 67). 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 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 critical test the next generation of highly sensitive diagnostics for rickettsioses.

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 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 non-invasive 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.

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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.[1]

				% clinical sensitivity	
Sample	**rickettsia**	**method**	**no. 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)	(43, 44)
	TGR	real-time PCR	257	5 (3-7)	(43, 44)
	SFGR	nested PCR	29	31 (31-31)	(53)
	SFGR	conventional PCR	179	69 (61-80)	(53, 54)

[1] Derived from studies for which serologic and PCR results on >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 *Rickettsia*; TGR – assays that target typhus group *Rickettsia*; no. assays column includes total number of assays reported, including some on the same samples but different approaches or targets.

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%	(68)
	IFA IgM	70-87%	84-100%	(16, 17, 68)
	ELISA IgG	80-97%	89-98%	(68-71)
	ELISA IgM	84-100%	73-99%	(68, 72)
	ImmChrom IgG RDT	86-95%	96-100%	(38, 68, 73)
	ImmChrom IgM RDT	82 - 94%	86-100%	(35, 38, 40, 68, 73)
	Dot EIA	60-100%	94-99%	(36, 68, 74)
Spotted fever rickettsiosis	IFA IgG	85-100%	99-100%	(60, 62, 75)
	IFA IgM	83-85%	100%	(62, 75)
	ELISA IgG	83%	87%	(61, 62)
	ELISA IgM	98%	94%[1]	(62)
Murine typhus	IFA IgG	≥ 83%	≥ 93%	(60)
	IFA IgM	53 - 85%	99%	

[1] Increasing data suggests lower specificity (56, 59, 62).

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