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REVIEW ARTICLE

Scrub Typhus Diagnostics: The Present and the Future

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Abstract

Scrub typhus, caused by *Orientia tsutsugamushi*, is a vector-borne, zoonotic disease commonly occurring in a geographic region known as the 'tsutsugamushi triangle'. Scrub typhus causes acute undifferentiated febrile illness (AUFI) with non-specific clinical features and is difficult to diagnose when a highly characteristic but not pathognomonic eschar is absent. Because the eschar is not always present, laboratory tests are required for diagnosis. Serological assays have been the mainstay of laboratory diagnosis of scrub typhus to date. Here, we present the major clinical features and clinical algorithms suggestive of this disease, to aid in better selection of diagnostic methods. The advantages and disadvantages of various scrub typhus assays are also discussed. Furthermore, we describe diagnostics, including serological and molecular assays, that may be available in the near future.

Keywords: *Orientia tsutsugamushi*, pathogenesis, eschar, culture, serology, molecular assays

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INTRODUCTION

Scrub typhus, a neglected febrile illness in the past, is now re-emerging in various regions of the world [1-4]. It is caused by the obligate intra-cellular bacterium *Orientia tsutsugamushi*, whose classification is described in Figure 1. Infections are transmitted by the larvae (chiggers) of the trombiculid mite [5]. Globally, chiggers of the genus *Leptotrombidium*, particularly *Leptotrombidium deliense*, are the most common vectors and reservoirs of *O. tsutsugamushi* [5-7]. Various rodent species serve as maintenance hosts, and humans are an accidental dead-end host [5,8].

Orientia was believed to be restricted to the geographic region known as the 'tsutsugamushi triangle' until the discovery of *O. chuto* in Dubai in 2006 [9] and *O.* *chiloensis* in Chile [2]. The tsutsugamushi triangle, which extends from far eastern Russia and Japan in the north/northeast, to Australia in the south and Pakistan in the west, is believed to be the major hotspot for this infection. Approximately 2 billion people living in that region are at risk [10], and 1 million cases are estimated to occur annually [11,12]. This incidence is considered to be an underestimate, because underdiagnosis of scrub typhus often occurs because the pathognomonic eschar is not always observed [13]. The eschar characteristically appears at the mite bite site as a painless black crust with minimal or no surrounding inflammation [14]. In the absence of the eschar, the diagnosis of scrub typhus is often missed [13]. Therefore, other acute febrile illnesses common in a given geographic region, such as enteric fever,
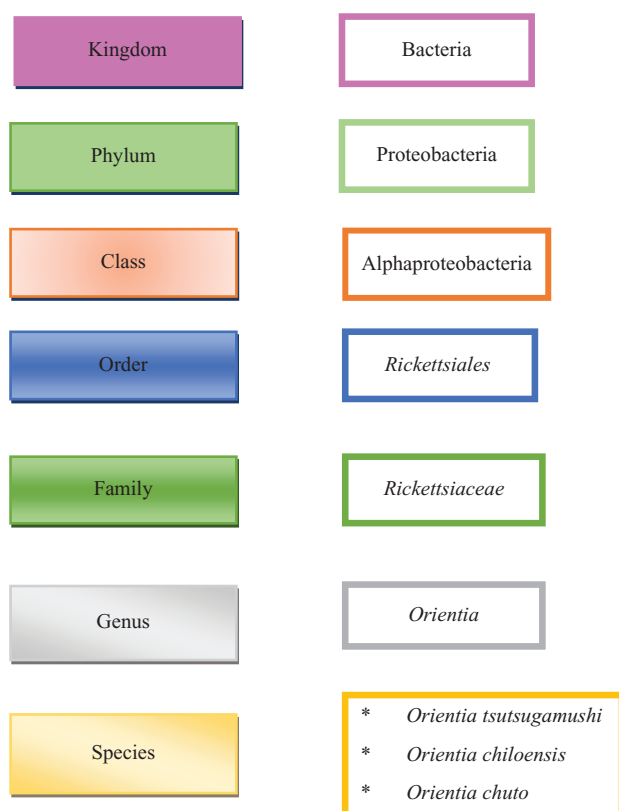
FIGURE 1 | Classification of *Orientia tsutsugamushi.*

dengue, leptospirosis, malaria, and spotted fever, must be ruled out through specific laboratory investigations [15]. Even in the absence of an eschar, certain clinical features and preliminary laboratory tests (blood picture) can alert physicians that a diagnosis of scrub typhus is highly probable (thus increasing the pre-test probability) [13]. Scrub typhus mortality rates vary from 6% to 30% [13,14] in untreated cases to 1.4% in treated cases [1]. Studies from Vellore in southern India have shown a decreasing trend in mortality from 14% in 2002–2003 to 1.8% in 2015–2017, which has been attributed to heightened clinical suspicion [14]. Delays in diagnosis also adversely affect outcomes, as demonstrated by an analysis of 93,481 cases and 57 deaths in China by Xin et al. in 2019. In that study, the mortality rate was 2.2 times higher when the time of diagnosis (the interval between disease onset and specific diagnosis) increased from 2 to 7 days [15]. Because this disease is vector borne, seasonality is observed [16-18], depending on the activity of the chigger vector [16].

O. tsutsugamushi's outer membrane contains the immunodominant 56 kDa surface protein, which composes 15% of the total protein of the organism. All individuals with scrub typhus develop antibodies to this antigen [19]. Moreover, the gene encoding the 56 kDa antigen has constant and variable regions, which can be used for molecular diagnosis and genotyping [20]. The common genotypes described are Kato, Karp, Gilliam, Boryong, TA716, TA763, TA686, and Kawasaki [21,22].

In the present review, current advances and practice in the diagnosis of scrub typhus are described. We also concisely discuss future diagnostic assays and their limitations, and provide several feasible suggestions.

PATHOGENESIS OF SCRUB TYPHUS

The organism has a predilection for endothelial cells, dendritic cells, monocytes, and macrophages. Figure 2 shows a simplified representation of pathogenesis process, which has been described in detail by Vincent [23] and Salje [24]. At the bite site, *O. tsutsugamushi* attaches to the host cell and is internalized, in a process facilitated by Sca C (a bacterial autotransporter protein); the TSA 56 antigen present on the organism; and syndecan 4 (a cell surface heparan sulfate proteoglycan), fibronectin, and integrin $\alpha5\beta1$ on the host cells. Once inside the cell, the organism is transported to the perinuclear area through dynein mediated transport on microtubules, then subsequently multiplies through binary fission. The organism is then released from the host cell through budding and begins to infect adjacent cells [23,24].

CLINICAL INDICATORS FOR SCRUB TYPHUS

In many hospitals in India, scrub typhus is one of the major differential diagnoses in individuals with acute undifferentiated febrile illness. A more elaborate definition of acute undifferentiated febrile illness includes individuals with a measured temperature $\geq 38°C$; a history of febrile illness of 2–14 days' duration; and no localized cause, as judged by the treating physician [25,26]. This illness can also be described by a fever of <14 days' duration with no evidence of a specific aetiology [27]. The prominent clinical indicators believed to be crucial for scrub typhus diagnosis include the following.

1. **Eschars**, scab formed at the mite bite site [10], are similar in appearance to a cigarette burn, comprising a black scab of approximately 1–1.5 cm surrounded by a minimal area of erythema [28]. Careful history taking and a thorough head to toe physical examination aids in the detection of the characteristic scrub typhus eschar [11,29,30]. Such examination is warranted [18,20] because eschars are more common in covered areas of the body [17]. In addition, diseases and conditions in which eschars have been described, including spotted fever spider bites, traumatic eschars, and anthrax, must also be ruled out [10,13,15,31]. Eschar rates in Southeast Asia and South Asia are highly variable, ranging from as low as 7% to as high as 97% [32]. The eschar detection rate from a tertiary care hospital in south India has been reported to improve from 10% (15 years ago) to 60% currently, as a result of thorough clinical examination and increased awareness of the disease among clinicians [17,33]. Eschar rates also depend on the infecting genotype [34] Moreover, lymphadenopathy has been reported to serve as a marker of eschar in many cases [35].

2. **Rash** has been described in some cases in other southeastern and eastern Asian countries [36]. Rash is uncommon
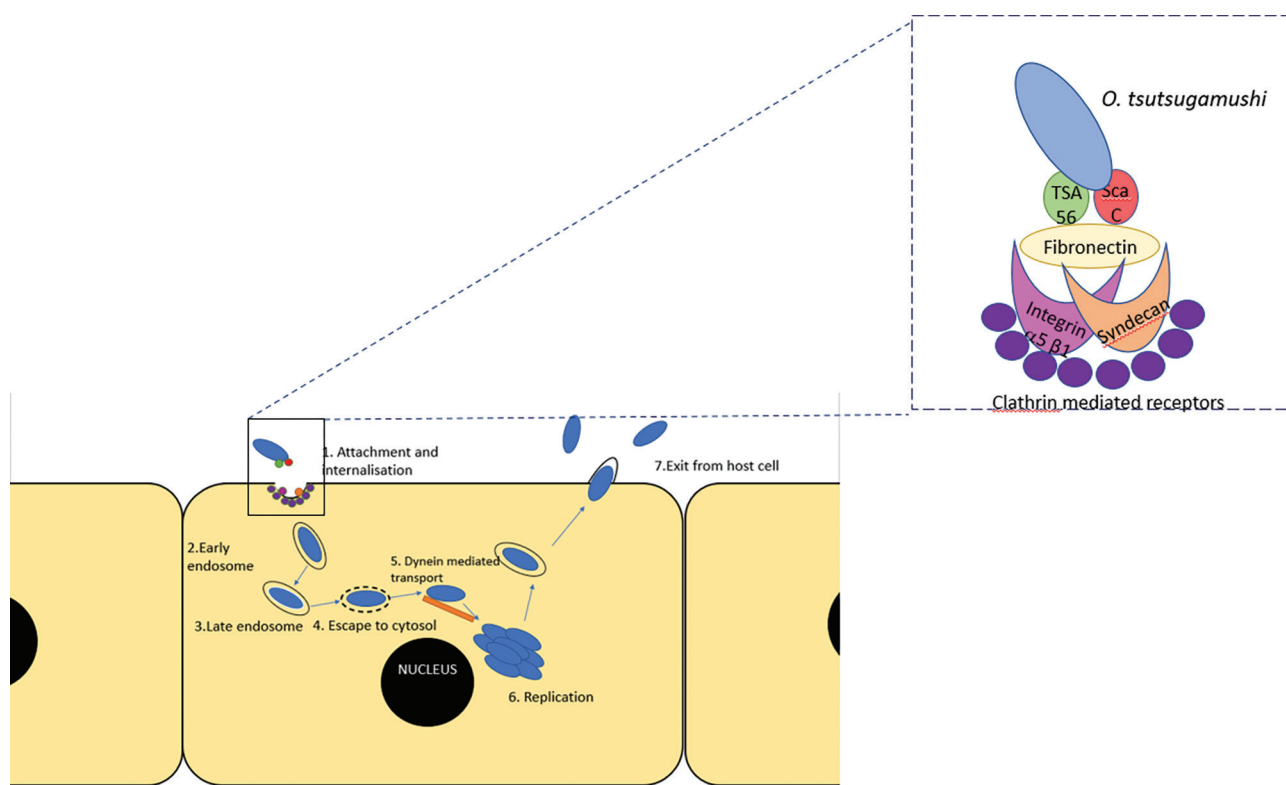
FIGURE 2 | Pathogenesis of scrub typhus.

in southern India but quite common in northern India. The rash in scrub typhus is classically described as a maculopapular rash and is difficult to identify in hyperpigmented individuals [29,30].

Laboratory parameters indicative of scrub typhus: platelet counts and ALT levels can alert clinicians to order scrub typhus testing, and procalcitonin can serve as a prognostic marker

1. **Platelet count**: Thrombocytopenia is a prominent finding leading to complications in scrub typhus. This feature is also seen in AFI due to dengue. Platelets clump together in EDTA samples, thus resulting in pseudothrombocytopenia, which is eliminated in heparinized samples [37,38]. When the platelet count (automated vs manual) is below 50,000 in a case of AFI, a manual platelet count must be ordered (*Gunasekaran K, personal communication)*. In dengue, the automated and manual values are often the same, whereas in scrub typhus, the manual value may be higher than the automated value, because the manual counting method is not affected by clumping of platelets [37].

2. **Alanine aminotransferase (ALT):** An ALT value above 100 U/L plays an important role in determining the prognosis of scrub typhus [39,40]. In a study in Vellore by Varghese et al., elevated transaminases (more than twice the normal levels) have been significantly associated with scrub typhus; this finding was observed in 90% of cases [41]. Su et al., in 2016, compared 288 scrub typhus cases vs 56 non-scrub typhus cases in Korea, and reported that elevated serum amino transferase had

an adjusted odds ratio of 3.75 for scrub typhus. They considered serum transferase to be elevated when AST was > 40 U/L and/or ALT was > 100 U/L [42]. Lee et al., in a study of 104 patients with acute hepatitis and 197 patients with scrub typhus, have observed ALT and AST levels >500 U/L only in acute viral hepatitis [43]. ALT and AST levels therefore can be used to guide the ordering of specific tests (scrub typhus serology and/or molecular assays) to identify the aetiology of suspected scrub typhus.

3. **Procalcitonin**: Elevated procalcitonin levels indicate severe infection in laboratory confirmed scrub typhus and are associated with elevated mortality [44].

SCORING SYSTEM FOR DIAGNOSIS

A clinical risk scoring system based on the World Health Organization's case definition has been devised and validated by Siriwongpan and others in Thailand and found to correctly identify approximately two-thirds of severe cases [45,46]. This algorithm has recently been evaluated in Manipal, India, by Gulati et al. in 198 confirmed cases. The authors have reported an improved prediction of severity: approximately 77% of cases were correctly classified as severe [47]. An algorithm to differentiate scrub typhus from dengue has been described by Mitra et al., by using the variables of oxygen saturation, total white blood cell count, haemoglobin, total bilirubin, serum glutamic/oxaloacetic transaminase (aspartate aminotransferase), and altered sensorium. A score < 13 suggests scrub typhus

with a sensitivity and specificity of 85% and 77%, respectively [48].

An eight point scoring system designed and evaluated by Jung et al. has shown a sensitivity and specificity exceeding 90% in Korean patients with a score of ≥4 [49]. For acute undifferentiated febrile illness in patients in South and Southeast Asia, if the fever responds to doxycycline therapy within 48–72 hrs, rickettsial infection, most probably scrub typhus, is often assumed [50]. Approximately 60% of patients with scrub typhus have been described to have an eschar, and cases presenting without an eschar are classified as having non–eschar related rickettsial diseases [29,30].

Scrub typhus global epidemiology has been extensively reviewed by Xu et al. [12] and Kala et al. [7], and its clinical spectrum has been described in detail by Devasagayam et al. [51]. Scrub typhus is clearly not restricted to the tsutsugamushi triangle, and scrub typhus like illness has been detected in regions outside the tsutsugamushi triangle. Because the disease has non–specific clinical features, and diagnosis can be easily missed, laboratory investigations play major roles in the diagnosis and treatment of scrub typhus [1-4]. This review focuses on the currently available methods for diagnosis and future prospects, particularly for rapid confirmation of scrub typhus.

LABORATORY DIAGNOSIS

Even in the presence of an eschar and the demonstration of a response to rickettsia specific therapy, scrub typhus diagnosis requires confirmation through laboratory testing. Traditionally, four methods have been used to confirm the etiological agent of an infectious disease or syndrome: isolation of organisms, antigen detection, nucleic acid amplification tests (NAATs), and antibody detection assays [7]. In Table 1, a brief overview of the non–serological assays is provided.

ISOLATION OF THE ORGANISM

Isolation of the pathogen *O. tsutsugamushi* is performed through cell culture or animal inoculation. This organism cannot be cultured in standard media, because it is an obligate intracellular pathogen [64]. These bacteria lack biosynthetic pathways, thus hindering their energy metabolism and consequently making them dependent on the host cell for their ATP supply [65].

The cell lines frequently used to cultivate *Orientia* species are L929 (mouse fibroblast cells) and Vero (African green monkey kidney) cells; other cell lines include HeLa cells and BHK-21 (baby hamster kidney cells) [66]. Some chigger cell lines, such as *Leptotrombidium chiangraiensis* and *Leptotrombidium imphalum* are also used to grow *O. tsutsugamushi* [67].

Isolation of organisms in culture, the gold standard method, can aid in the characterization of isolates and is particularly useful for understanding an organism's biology and immunopathogenesis. Furthermore it provides a source of antigens for serological assays, particularly immunofluorescence assays (IFAs), the current serological

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TABLE 1 | Advantages and disadvantages of non-serological assays for scrub typhus diagnosis.

Method/assay	Advantages	Disadvantages	References
Pathogen isolation (cell culture or animal inoculation)	Gold standard test Definitive evidence as etiological agent 100% specific Useful for characterization and confirmation of new species or strains	Labor intensive and time consuming Requires trained personnel Requires BSL3/ABSL3 containment facility Clinically not useful (median time to detection of 27 days) Requires PCR and IFA for confirmation Low sensitivity (≤50%)	[3,52-55]
PCR (cPCR, nPCR, and qPCR)	Positive results are confirmatory Sensitive and specific: acute phase Real-time results: qPCR Phylogenetic analysis: cPCR and nPCR LOD (10–50 copies/reaction)	Requires technical expertise Requires expensive reagents and equipment Contamination issues, particularly with nPCR Best yield: acute phase (Max: two weeks) Specific treatment reduces sensitivity	[7,10,56-58]
LAMP and RPA	Isothermal reaction Sensitive and specific, depending on the primers Can be performed on various specimens LOD (comparable with nPCR and qPCR)	Requires technical expertise Requires expensive reagents Requires extensive validation High yield only during the first 2 weeks (acute phase) Specific treatment reduces sensitivity	[7,57,59-62]
IHC and IIP	Can be performed on eschar biopsies and other specimens Rapid detection, in contrast to culture methods	Requires technical expertise Expensive, requires fluorescence microscope	[63]

BSL – Biosafety Laboratory, PCR – Polymerase chain reaction, IFA – Immunofluorescence assay, cPCR – Conventional polymerase chain reaction, nPCR – Nested polymerase chain reaction, qPCR – Qualitative polymerase chain reaction, LOD – Limit of detection, LAMP – Loop mediated isothermal amplification, RPA – Recombinase polymerase amplification reaction, IHC – Immunohistochemistry, IIP – Indirect immunoperoxidase.

gold standard [68]. However, isolation is a laborious process requiring trained personnel, appropriate infrastructure, and a BSL3 containment facility. This process is also highly time consuming , on an average being 28 days [66] and requires other methods, including immunohistochemistry or polymerase chain reaction (PCR), for confirmation [7].

To shorten the turnaround time of culturing for isolation of *O. tsutsugamushi* in cell culture from weeks to days, shell vial culture has been proposed by Santibanez et al. [66]. Implementation of shell vial culture decreases the time to positivity (i.e., the time required for isolation in culture) to 48–72 hrs for spotted fever group rickettsia [69-71]. Although isolation of *O. tsutsugamushi* is the gold standard (100% specific), its sensitivity is approximately 35-50% [10].

MOLECULAR ASSAYS

Specimens for NAAT assays may include eschars, eschar swabs, skin biopsies of eschars or rashes, whole blood, buffy coat, and blood clots [72,73]. Whole blood sample positivity is markedly diminished within 24 hours after initiation of therapy [10], Eschar or rash biopsy specimens can yield positive results even after treatment has been initiated and therefore are the specimens of choice for molecular confirmation of scrub typhus [2].

Various NAATs are available for laboratory confirmation of scrub typhus, including PCR (conventional, nested, or real time), loop mediated isothermal amplification (LAMP), and recombinase polymerase amplification reaction (RPA). All molecular assays require costly special reagents and labware for detecting *O. tsutsugamushi* specific DNA [74]. LAMP and RPA are isothermal reactions that can be performed with low cost heating blocks, whereas conventional and real time (TaqMan and SYBR green) PCR requires expensive equipment with high maintenance costs. Dedicated, well demarcated laboratory space is essential for providing reliable results, including separate spaces or rooms for nucleic acid extraction, preparation of the master mix (clean room), sample addition, nucleic acid amplification (thermal cycling), and gel electrophoresis for conventional and nested PCRs. Availability of dedicated, physically well separated spaces is necessary to prevent contamination of specimens and reagents, and to ensure reliable and reproducible results when NAATs are used for diagnosis [75]. In practice, real time PCR (qPCR), particularly the TaqMan method, is relatively less prone to contamination, easier to perform, and gives faster results, thus providing benefits over other assays [56].

Specific molecular assays for detection of *O. tsutsugamushi* have been described. The targets for these molecular assays include the *htrA* (47 kDa periplasmic serine protease), 56 kDa type-specific antigen, *rrs* (16S rRNA), and *groEL* (heat shock protein Hsp60) genes [5,7]. The most commonly used assay detects the 47 kDa antigen gene through qPCR; however, amplification of the 56 kDa antigen

gene by nested PCR coupled with Sanger sequencing provides information on the prevalent genotypes in a given geographic area [76].

PCR is most often positive in the early phase of illness (until the 10th day), although samples have tested positive even on the 22nd day of illness [77]. The LAMP assay, an isothermal assay, is simple, relatively inexpensive, and applicable in resource limited settings. The gene targets used are the 47 kDa antigen and GroEl. The major drawback of this method is that it requires a laborious standardization process [7,9,56].

Newer molecular assays are available, such as TrueNat Scrub T, a chip-based qPCR test for the quantitative detection of scrub typhus bacteria in blood/serum/plasma specimens. The target sequence for this assay is the *htrA* gene of *O. tsutsugamushi*. The test results indicate that scrub typhus is either 'detected' (positive) or 'not detected' (negative). In positive cases, the CT value is displayed on the screen. The limit of detection has been found to be 339 copies/mL. The major disadvantage of this method is that the evaluation is performed without testing samples from patients with malaria, dengue, enteric fever, or sepsis, all of which are differential diagnoses for scrub typhus [78]. The major advantages of nucleic acid detection tests are their speed, sensitivity, and specificity depending on the primers. They have a low limit of detection, and can detect both viable and non-viable organisms with a high yield in the first 2 weeks of infection. Their drawbacks, which are common to all molecular assays, is that they are labour intensive, require skilled operators, and are performed in batches with long turnaround times in practice and with high chances of contamination [7]. Nested PCR involves two rounds of PCR and hence is the most prone of the PCR assays to contamination. TaqMan qPCR (primers and probes) provides more rapid results in real time, but the fidelity of amplification cannot be verified by sequencing. In contrast, conventional PCR, nested PCR, and even SYBR green PCR amplicons can be Sanger sequenced to verify the amplified fragments, thus providing information on the circulating genotype.

ANTIBODY DETECTION (SEROLOGICAL ASSAYS)

Detection of pathogen specific antibodies in patient blood samples may indicate current or recent infection or past infection/exposure, depending on the type of antibodies detected. Antibody detection in serum, plasma, or whole blood is the mainstay of diagnosis for scrub typhus [79].

Detection of IgM in patient serum is considered to indicate a recent or ongoing infection, whereas IgG detection indicates past infection by the infectious agent. IFA is the serological reference standard and is considered the gold standard for diagnosis by many [57]. By definition, definitive diagnosis through serological means should demonstrate a rise in titre or seroconversion [80]. Serological assays including IFA can yield false negative results early in

the illness, because IgM antibodies are rarely detected (< 5 days), whereas molecular targets specific to *O. tsutsugamushi* can be detected [68]. A practical difficulty is determining serological assay cut offs, which should be tailored to the specific geographic region and the other acute undifferentiated febrile illnesses that are prevalent in the same area and present similar clinical pictures [81]. The 56 kDa antigen is immunodominant and is the most abundant outer surface protein of *O. tsutsugamushi*. All individuals infected with *O. tsutsugamushi* develop an antibody response to this antigen. However, owing to genotype variations, a genotype specific antibody response is developed; therefore, knowledge of circulating genotypes is necessary to enable use of the appropriate antigen in the test [82]. Because all genotypes cannot be incorporated into diagnostic tests, the inclusion of region-specific genotypes of the 56 kDa antigen is critical for diagnosis. The various serological assays are listed in Table 2, and their advantages and disadvantages are briefly described in Table 3.

The best performance of serological assays for scrub typhus occurs during the second week of illness. The sensitivity is lowest for the Weil Felix test, reaching a maximum of 50%. The specificity has also been reported to be low [93], although we have consistently observed a specificity above 90% [14]. A diagnosis of scrub typhus can be confirmed serologically by demonstrating a fourfold rise in titre; paired samples (acute and convalescent, taken at least 2 weeks apart) must be tested [94]. Although this method can effectively confirm the diagnosis and has great epidemiological value, it is always retrospective and therefore has little clinical utility. Serological diagnosis is almost always performed on acute specimens. Therefore, determining the cut off value (clinically significant value) for each geographical area is important, given that re-infection is quite common [68,95]. The clinically significant value or titre can be robustly detected by testing known positive samples, samples from healthy individuals, and samples from individuals with diseases with clinical features (particularly eschar negative) similar to scrub typhus. Readers are referred to extensive reviews on this topic for further guidance [57,68,95]. The overall sensitivity and specificity of the nonculture assays are described in Table 4.

TABLE 2 | Details of assays available for serodiagnosis of scrub typhus.

Parameters	Weil Felix [3,7,83]	ELISA [3,84,85]	IFA [68,86]	RDT [87-90]
Antibodies detected (predominant)	IgM	IgM/IgG	IgM/IgG and total	IgM/IgG
Principle	Agglutination	ELISA	Immuno-fluorescence	Lateral flow assay
Sample type	Serum	Serum	Serum	EDTA/whole blood/serum
Infrastructure	+	+++	+++	+
Operator skill	+	+++	+++	+
Automation	-	+++	-	-
Batch testing	No	Yes	Yes	No
Observations	Subjective	Objective	Subjective	Subjective
TAT*	12–24 hrs	>24 hrs	>24 hrs	<30 mins
Results	Semi-quantitative (titre)	Qualitative (positive/negative)	Semi-quantitative (titre)	Qualitative (positive/negative)
Inter and intra observer variation	+	-	+++	+
Antigens used	Heterophile (*Proteus spp.*)	Recombinant 56 kDa	Whole organism + host cell antigens	Recombinant 56 kDa
Definitive diagnosis (paired sera)	Rise in titre	Sero-conversion	Sero-conversion and rise in titre	Seroconversion
Cross reactions	+++	++	++	+
Use in sero-surveillance	+	+++	+	+
Cost effectiveness	++	+++	+	+

ELISA – Enzyme linked immunosorbent assay, IFA – Immunofluorescence assay, RDT – Rapid diagnostic test, IgM – Immunoglobulin M, IgG – Immunoglobulin G.
***Turnaround time (TAT) – Includes both time for testing and result reporting.

TABLE 3 | Advantages and disadvantages of various serological assays.

Assay	Advantage	Disadvantage	References
IFA	IgM and IgG antibodies detected Serological gold standard Semi-quantitative Rise or fall in titre can be demonstrated by serial testing	Expensive; fluorescence microscope needed Requires technical expertise and standardization Cross reaction with other rickettsial species Labour intensive Subjective: inter-observer and intra-observer variation Requires paired sera for definitive diagnosis	[8,68,86]
ELISA	Objective, economical, and technically simple With automation, large numbers of samples can be tested Assay can be automated Minimal inter-assay and intra-assay variation Useful for surveillance studies Can detect lower levels of antibodies than IFA	Requires instrumentation: ELISA reader or ELISA workstation Batch testing (single samples cannot be tested) Region wise cut-off values must be defined ELISA is qualitative Rise in titre difficult to demonstrate	[84,88]
RDTs	Rapid turnaround time; applicability in field settings Simple to perform (point of care testing) Specialized equipment not required	Chances of error due to prozone phenomenon (because no dilution is performed) False positive results high if not properly validated Indeterminate results due to faint bands	[83,89-91]
Weil Felix	Economical Semi-quantitative Paired sera: rise in titre demonstrated Specificity: moderate to good Easily performed and commonly used in resource limited settings	False positives result from to cross-reactivity with other rickettsial organisms and *Proteus* infections, particularly UTI Subjective results in inexperienced hands Low sensitivity (~50%)	[3,92]

ELISA – Enzyme linked immunosorbent assay, IFA – Immunofluorescence assay, RDT –Rapid diagnostic test, IgM – Immunoglobulin M, IgG – Immunoglobulin G, UTI – Urinary tract infection.

TABLE 4 | Sensitivity and specificity of non-culture diagnostic assays for scrub typhus.

Method	Method/assay	Target/platform	Sensitivity	Specificity	References
Molecular diagnostics	Conventional PCR	47 kDa 56 kDa GroEL 16S rRNA	3–7% 0–96% 66% 45%	100% 100% 100% 87%	[56,96,97]
	Nested PCR	47 kDa 56 kDa GroEL 16S rRNA	81–85% 16–100% 90.4% 20.6%	100% 88–100% 100% 100%	[56,77,98,99]
	Real time PCR	47 kDa 56 kDa GroEL 16S rRNA	63–81% 65–73% 56.4% 52%	90–100% 100% 96.2% 100%	[56,100,101]
	LAMP	47 kDa GroEl	52% 87.5%	94% 100%	[55,62,102]
	RPA	47 kDa	86%	100%	[59,60]
Serological tests	IFA	Karp, Kato, and Gilliam	78–97%	98–100%	[8,68,86]
	ELISA	56 kDa antigen cocktail	70–100%	87–100%	[10,57,85,92]
	RDTs	Single/mixture of recombinant 56 kDa proteins	61–100%	74–100%	[89-91]
	Weil Felix	*Proteus spp.* antigens (OX-K positive)	16–60%	96–98%	[3,57,92,103]
Antigen detection	IHC/IIP	Mouse polyclonal anti-*Orientia tsutsugamushi* antibody	65%	100%	[63]

PCR – Polymerase chain reaction, IFA – Immunofluorescence assay, LAMP – Loop mediated isothermal amplification, RPA – Recombinase polymerase amplification reaction, ELISA – Enzyme linked immunosorbent assay, IFA – Immunofluorescence assay, RDT – Rapid diagnostic test, IHC – Immunohistochemistry, IIP – Indirect immunoperoxidase test.

FUTURE ASSAYS

IgM capture ELISA

All commercial IgM ELISA methods for detecting scrub typhus use indirect antibody detection, wherein the antigen is coated on a solid support, serum is added, and bound IgM is detected with anti-human IgM conjugate. This method often has drawbacks of both false positivity and false negativity, because of rheumatoid factors and the presence of high levels of IgG. The drawbacks of indirect IgM detection can be most effectively resolved by using IgM antibody capture (MAC) ELISA, which is routinely applied for the detection of anti-viral IgMs. One report has described IgM capture ELISA for the detection of IgM against *O. tsutsugamushi*, and no commercial products are currently available [104]. A well optimized MAC ELISA for *O. tsutsugamushi* is expected to aid in early and accurate diagnosis of scrub typhus.

MULTIPLEX PCRS

Multiplex PCR targets many genes (47 kDa antigen and the GroEl protein) and has been found be more sensitive (86%) and specific (100%) than the other molecular assays [105].

MULTICOPY GENE VS SINGLE COPY GENE ASSAYS

Compared with 47 kDa antigen qPCR, *traD* gene qPCR detects more cases. The *traD* gene has also been reported to be specific to *O. tsutsugamushi*. In this qPCR, multiple alleles of the *traD* gene (the conjugative transfer protein D gene, encoding a subunit of the type IV secretion system) are amplified. Single copy gene amplification yields high threshold cycle values. A preliminary evaluation has suggested that the copy number of *traD* varies among strains of *O. tsutsugamushi*, ranging from 32 to 1000 copies [106]. Single copy qPCRs typically require more than ten genome copies per reaction to yield a positive signal, whereas amplification is consistently achieved with *traD* for 0.1 genome copies/reaction [107]. Furthermore, Prakash et al. have observed that qPCR for *traD*, compared with the 47 kDa antigen, is more sensitive in detecting *O. tsutsugamushi* in chiggers [108].

As newer species of *Orientia* are discovered, a genus specific qPCR such as that described by Jiang et al. (2022) may be useful. This 16S rRNA based qPCR detects a 94 bp region common to the three *Orientia* species (*O. tsutsugamushi*, *O. chiloensis*, and *O. chuto*). Laboratory evaluation has confirmed the utility of this assay, which detected all 47 Orientia positive specimens, including five *O. chiloensis* and one *O. chuto*, while yielding negative findings for 22 rickettsia species, 11 unrelated bacterial specimens, one human DNA sample, and four mouse DNA samples. Furthermore, a field evaluation has confirmed the utility of this method, which correctly identified 28 cases, as confirmed by nested PCR [109].

CHEMILUMINESCENCE IMMUNOASSAYS

Chemiluminescence immunoassays (CLIAs) often use antigen/antibody coated paramagnetic particles for detection, and have higher sensitivity and specificity than ELISA [87,110]. Batch testing is not necessary, because samples can be tested as they arrive in the laboratory, thus making on demand testing a reality. Furthermore, even small concentrations of biological analyte in samples can be detected [111]. The primary advantage of CLIA is its wide dynamic range, which may aid in setting appropriate test cut-offs [111]. Currently, CLIA has been found to be very useful for laboratory diagnosis of HIV, hepatitis B [112], adenovirus [113], HCV, syphilis [114], *T. gondii* [115], *Varicella Zoster* [116], and *West Nile virus* [117].

CELL BASED IFA

Cell based IFA has been effectively used in autoimmune disorders [118], and the same technique can be applied for infectious diseases including scrub typhus. Creation of a chimeric antigen becomes necessary, because of the numerous genogroups and genotypes. TSA of *O. tsutsugamushi* 56 kDa antigen is the major outer membrane protein and the major determinant of *O. tsutsugamushi* serotypes [119]. A baculovirus expression system and transfected *Spodoptera frugiperda* (Sf9) have been used for expression of the TSA core region. The rTSAs so generated were used for IFA in the diagnosis of scrub typhus. Preparation of *O. tsutsugamushi* infected cells requires a BSL3 facility, but recombinant antigens can be prepared in a BSL2 laboratory. The rTSA based IFA (using transfected Sf9 cells) is cost effective and performs as good as the IFA using *O. tsutsugamushi* infected cells [120]. We are currently developing a cell based IFA for scrub typhus.

FLUORESCENT NANOPARTICLE BASED LATERAL FLOW IMMUNOASSAY

Conventional lateral flow assays (LFAs), often referred to as rapid diagnostic tests (RDTs), use either colloidal gold or coloured polystyrene particles as tracer molecules to allow for visual readout of the test with the naked eye [121,122]. Saraswati et al. have extensively reviewed the performance characteristics of these point of care tests. Their meta-analysis has suggested that these tests have a sensitivity of 0.37–0.86 (mean 66%) and a specificity of 0.83–0.97 (mean 92%) [64]. Moreover, visual LFA results in subjectivity in reading the results and prevents assigning of any cut-offs, given that the results are binary, i.e., test line observed or no line observed [123]. These drawbacks of conventional LFA can be solved by using high sensitivity fluorescent nanoparticles, particularly upconverting phosphor nanoparticles (UCNPs), instead of gold or coloured polystyrene particles. Salminen et al. have demonstrated that replacing colloidal gold with UCNPs enhances the analytical sensitivity of malaria RDT by more than 100 fold [124].

Assays to detect *Yersinia pestis*, *Bacillus anthracis* spores, and *Brucella spp.* have been evaluated in China and found to have a detection limit of 10^5 CFU/ml [125]. Li et al. have developed and evaluated a UCNP based assay for detection of anti-*Trichinella spiralis* IgG antibodies in pig serum. This assay is highly promising and has shown very good concordance with the gold standard [126]. Similar advancements in scrub typus detection may be highly useful for rapid diagnosis of scrub typhus.

NEXT GENERATION SEQUENCING

Recently, next generation sequencing (NGS) on clinical samples and metagenomic NGS (mNGS) have been used for identifying disease aetiology when conventional techniques have been unable to identify the pathogen. NGS in various biological samples has successfully detected *O. tsutsugamushi* [127-132]. A summary of cases for which NGS has been used is described below.

NGS has detected *O. tsutsugamushi* (317 reads) in the plasma on day 16 of illness in a 76-year old Chinese patient with fever and kidney injury, who was eschar negative. Subsequent plasma PCR was positive [127]. A 51 year old female farmer from Guangzhou (a scrub typhus endemic area) has been diagnosed with scrub typhus through NGS on the 12th day of fever (377 reads) followed by PCR confirmation, [128]. In a patient with fever (no eschar), myalgia, and UTI, scrub typhus has been identified by detection of seven *O. tsutsugamushi* reads by NGS on day 13, after serological tests for various bacteria (not scrub typhus) and viruses yielded negative results [129]. NGS has identified 9 of 13 patients with suspected scrub typhus in Ganzhou, China, one of whom was positive according to the Weil-Felix test and another of whom was positive according to qPCR. The number of *O. tsutsugamushi* specific reads ranged from 2 to 460 [130]. NGS on a blood sample collected on the 11th day of fever has shown 226 reads corresponding to *O. tsutsugamushi* in a 51 year old botanical garden worker. Scrub typhus qPCR performed subsequently was also positive, thus confirming the aetiology [131]. In a patient in Jiangxi, China (a scrub typhus endemic area), 6 of 16,422,429 reads from alveolar lavage fluid and 22 of 15,608,629 reads from blood were similar to *O. tsutsugamushi* [132]. Interestingly, IFA or ELISA for scrub typhus was not performed on any of the above patients who were diagnosed with scrub typhus through NGS [127-132]. In all these patients, NGS was performed in the second week of illness, when IFA or ELISA findings are likely to be positive [14].

Currently, mNGS is not available in most hospitals, particularly primary and secondary care centres. Hence, testing must be outsourced, and reports are not available immediately. Even if mNGS is available, it cannot be performed on demand, and requires a minimum of 2–3 days, as well as expertise for analysis and interpretation [127-132]. We believed that mNGS currently has a limited role in diagnosis, because most cases occur in patients who report to centres with very limited resources for diagnosis.

In summary, the 56 kDa antigen determines the genotype, which is directly associated with variation in the four variable domains. Because genotypes differ across countries and even regions, knowledge of the circulating genotypes is essential for designing serological and molecular assays. Therefore, researchers developing scrub typhus diagnostics should be aware of the circulating genotype to design region specific assays.

CONCLUSION

Scrub typhus causes severe illnesses in people worldwide: the pathogen *O. tsutsugamushi* been found to cause infections outside the 'tsutsugamushi triangle'. Adding to this concern is the growing number of misdiagnosed or underdiagnosed cases, given that the symptoms for this illness are vague and non-specific. Some challenges in diagnosing *O. tsutsugamushi* in laboratories are the requirements of BSL3 containment, skilled personnel, and specialized equipment. Serology is the mainstay of diagnosis of scrub typhus, and IFA is the serological reference standard. However, this test has drawbacks of intra- and interobserver variation (subjectivity), high cost, and limited availability of the necessary infrastructure.

Currently, a combination of clinical features, molecular (47 kDa antigen qPCR) and serological assays (IgM ELISA) are used to confirm the diagnosis in suspected cases. In resource poor centres, rapid diagnostic assays combined with clinical features are most effective. Therefore, we expect that serological assays, which are relatively objective and easier to perform in resource limited settings, will become available in the future.

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