Scrub typhus diagnostics: An overview

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Abstract:

Scrub typhus caused by Orientia tsutsugamushi is a vector-borne, zoonotic disease which is common in the tsutsugamushi triangle. Scrub typhus causes AUFI in patients with non-specific clinical features and is difficult to diagnose when the highly characteristic but not pathognomonic eschar is not seen. As the eschar is not always present, laboratory tests are needed for confirmation. Serological assays are the main stay of laboratory diagnosis of scrub typhus thus far. In this review, we present the salient clinical features and clinical algorithms suggestive of this disease that help in better selection of diagnostics. The various scrub typhus assays along with their advantages and disadvantages have also been discussed. Furthermore, we have also discussed the upcoming diagnostics, both serological and molecular assays which can be available in the near future.

Introduction

Scrub typhus, a neglected febrile illness of the past is now re-emerging in various regions of the world (1–4). It is caused by an obligate, intra-cellular bacteria *Orientia tsutsugamushi*, the classification of which is described in Figure 1. It is transmitted by the larva (chiggers) of trombiculid mite (5). Globally, chiggers of the genus *Leptotrombidium* especially *Leptotrombidium deliense* are the commonest vectors and reservoirs of *O. tsutsugamushi* (5–7). Various rodent species act as the maintenance host and man is an accidental dead end host (5,8).

Orientia was thought to be restricted to the "tsutsugamushi triangle" till the discovery of *Candidatus O.chuto,* in Dubai in 2006 (9) and *Candidatus O.chiloensis* in Chile (2). The tsutsugamushi triangle which extends from far eastern Russia and Japan in the north/ north-east, Australia in the south, and Pakistan in the west, is said to be the major hotspot for this infection. Around 2 billion people living in that region are at risk (10) and about 1 million cases occur annually v (11,12). This incidence is considered an underestimate due to under diagnosis of scrub typhus, as the pathognomonic eschar is not always seen (13).The eschar characteristically appears as a painless black crust with minimal or no inflammation surrounding it and occurs at the mite bite site (14). When absent, the diagnosis of scrub typhus is often missed (13). Therefore, other acute febrile illnesses common in the region like enteric fever, dengue, leptospirosis, malaria, spotted fever etc., need to be ruled out by specific laboratory investigations (15). Even in the absence of an eschar, certain clinical features and preliminary laboratory tests (blood picture) can alert the physician that diagnosis of scrub typhus is highly probable (increase the pre-test probability)(13). The mortality rates can vary from 6% to 30% (13,14) in untreated cases to 1.4% in treated cases (1). Studies from Vellore, Southern India have shown a decreasing trend in mortality from 14% in 2002-03 to 1.8% in 2015-17 which has been attributed to heightened clinical suspicion (14). Further, a delay in diagnosis also adversely affects the outcome as demonstrated by an analysis of 93,481 cases and 57 deaths in China by Xin et al in 2019. They found that the mortality rate was 2.2 times higher when the time of diagnosis (interval between disease onset and specific diagnosis) increased from 2 to 7 days (15). As it is a vector borne disease, seasonality is observed (16–18) which depends on the activity of the chigger vector (16).

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

In the present review, latest advances, and practices in the diagnosis of scrub typhus are described. We also made an attempt to provide a concise note on the future diagnostic assays and their limitations along with some feasible suggestions.

Pathogenesis of scrub typhus

The organism has a predilection for dendritic cells, monocytes, and macrophages. Figure 2 describes the simplified representation of the pathogenesis which was described in detail by Vincent G (23) and Salje J (24). At the mite bite site, *Orientia tsutsugamushi* attaches to the host cell and is internalized. This is facilitated by Sca C (bacterial autotransporter protein) and 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 by dynein mediated transport on microtubules, and it multiplies there by binary fission. The organism after multiplication is then released out of the host cell by budding and starts infecting the adjacent cells (23,24).

Clinical indicators for scrub typhus

In many hospitals in India, scrub typhus is one of the top differentials in individuals with acute undifferentiated febrile illness (AUFI). A more elaborate definition of AUFI includes individuals having a measured temperature of ≥ 38 °C, a history of febrile illness of 2–14 days duration, with no localized cause as judged by the treating physician (25,26). Or it can also be described as individuals having fever for <14 days duration, with no evidence of specific etiology (27). The prominent clinical indicators which are thought to be vital for scrub typhus diagnosis include:

1. **Eschar** is the scab formed at the area of mite bite site (10). It appears as a cigarette burn with a black scab of around 1–1.5cm with a minimal area of erythema around it (28). A careful history and thorough physical examination (head to toe) helps in the detection of the characteristic scrub typhus eschar (11,29,30). A head to toe examination is warranted (18,20) as eschars are more common in the covered areas of the body (17). In addition, it also rules out diseases and conditions where eschar has been described - spotted fever, spider bite and traumatic eschars and anthrax (15) (10,13,31). Eschar rates in South east Asia & South Asia, were found to be 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 improved from 10% (15 years ago) to the current 60 %. This is attributed to thorough clinical examination and increased awareness of the disease among the clinicians(17,33). Eschar rates also depend on the infecting genotype(34) Lymphadenopathy has also been reported to serve as a marker for eschar in many cases (35).

2. **Rash** has been described in some cases in other South- eastern countries and eastern countries (36). In southern India it is uncommon, whereas in northern India it is quite common. The rash in scrub typhus is classically described as maculopapular rash (29,30).

Laboratory indicators for scrub typhus

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

2. **Alanine aminotransferase (ALT):** An ALT value of over 100 U/L, plays an important role in prognosis of scrub typhus (39,40). In a study published from Vellore by Varghese *et al*, elevated transaminases (> twice normal) was significantly associated with scrub typhus, as this finding was observed in 90% of the cases (41). Su *et al* 2016 from Korea compared 288 scrub typhus cases vs 56 non scrub typhus cases and reported that elevated serum amino transferase had an adjusted odds ratio (AOR) of 3.75 for scrub typhus. They considered elevated serum transferases when AST was > 40 U/L, and/ or ALT was > 100 U/L(42). Lee *et al*, reported that ALT and AST levels >500U/L were seen only in acute

viral hepatitis when they measured this in 104 acute hepatitis and 197 scrub typhus patients (43).

3. **Procalcitonin**: Elevated procalcitonin levels indicates a severe infection in scrub typhus and this was associated with higher mortality (44).

Scoring system for diagnosis

A clinical risk scoring system based on the World Health Organization (WHO) case definition was devised and validated by Siriwongpan and others in Thailand. They reported that their scoring system could correctly identify around 2/3rds of their severe cases (45,46).This algorithm has been recently evaluated in Manipal, India by Gulati *et al* on 198 confirmed cases. They report an improved prediction of severity as around 77% of the cases were correctly classified as severe cases (47). An algorithm to differentiate scrub typhus from dengue has been described by Mitra S *et al* using 6 variables like oxygen saturation, total white blood cell count, haemoglobin, total bilirubin, serum glutamic/oxaloacetic transaminase (Aspartate aminotransferase), and altered sensorium. A score of < 13 favoured scrub typhus with a sensitivity and specificity of 85% and 77% respectively(48).

An eight point scoring system designed and evaluated by Jung HC *et al* had a sensitivity and specificity of more than 90% in Korean patients at a score of ≥4 (49). For AUFI patients in south and south east Asia, if fever responds to Doxycycline therapy within 48-72 hrs, it is often assumed to be due to rickettsial infection, most likely scrub typhus (50). About 60% of scrub typhus patients were described to have an eschar, and cases presenting without an eschar are classified as non- eschar related rickettsial diseases (NERD) (29,30).

Global epidemiology has been extensively reviewed by Xu *et al* (12) and Kala D *et al* (51). The clinical spectrum of scrub typhus has been described in detail by Devasagayam *et al* (52). It is clear that scrub typhus is not restricted to the tsutsugamushi triangle and scrub typhus like illness has been detected from regions outside the tsutsugamushi triangle as well. As the disease has non-specific clinical features and diagnosis can be easily missed, lab investigations play a major role in the diagnosis and treatment of scrub typhus (1-4). So, in this review, we are concerning ourselves with the current methods available for diagnosis and the future prospects especially rapid confirmation of scrub typhus.

Laboratory diagnosis

Even in the presence of eschar and demonstration of response to rickettsia specific therapy, the confirmation of the diagnosis of scrub typhus requires laboratory testing. Traditionally four methodologies are available for confirming the etiological agent of an infectious disease or syndrome; isolation of organisms,) antigen detection, nucleic acid amplification tests(NAATs) and antibody detection assays (51). In Table 1, a brief overview of the non-serological assays has been provided. In Table 4, the performance of various non-culture tests/ assays in diagnosing the scrub typhus in clinical samples is mentioned.

Isolation of organism

Isolation of the pathogen *O. tsutsugamushi* is done by cell culture or animal inoculation. This organism cannot be cultured in the usual media as they are obligate intracellular pathogens(53). These bacteria lack biosynthetic pathways which hinders their energy metabolism which in turn makes them dependent on the host cell for their ATP supply(54).

The common cell lines used to cultivate *Orientia* species are L929 (mouse fibroblast cells) & Vero(African green monkey kidney) cells other cell lines include HeLa cells, BHK-21(baby hamster kidney cells)(55). Some of the chigger cell lines like *Leptotrombidium chiangraiensis* (Lc) & *Leptotrombidium impalum* (Li) are also used to grow *Orientia tsutsugamushi*(56).

Isolation of organism in culture is the gold standard can also help in characterization of the isolate. It is especially useful to understand the biology of the organism, immunopathogenesis, furthermore it acts as a source of antigens for serological assays especially IFA – the current serological gold standard(57). However, it is a laborious process requiring trained personnel, appropriate infrastructure and a BSL 3 containment facility. This process is also very time consuming (median time to detection is 27 days) and requires other methods like immuno-histochemistry, PCR for confirmation (51).

To improve the turnaround time, shell vial culture has been proposed as a rapid method for isolation of *O.tsutsugamushi* in cell culture by Santibanez et al (55).This is because implementation of the shell vial culture reduced the time to positivity (time for isolation in culture) to 48- 72 hrs for spotted fever group rickettsia (58–60). Though isolation of *Orientia tsutsugamushi* is the gold standard (100% specific) the sensitivity is around 35-50% (10).

Molecular Assays

Specimens for nucleic acid amplification tests(NAATs) assays include eschar, eschar swab, skin biopsy of eschar or rash, whole blood, buffy coat and blood clot (61,62). The whole blood sample positivity is drastically reduced within 24 hours of initiation of therapy (10), The eschars or rash biopsy specimens can give positive results even after treatment has been initiated thereby making it the specimen of choice for molecular confirmation of scrub typhus (2).

Various NAATs are available for laboratory confirmation of scrub typhus. They include polymerase chain reaction (Conventional, Nested, Real Time), Loop mediated isothermal amplification, and Recombinase polymerase amplification reaction. All molecular assays need dedicated space and special reagents and labware (which are costly) for detecting *Orientia tsutsugamushi* specific DNA (63) . LAMP & RPA are isothermal reactions and can be performed on low cost heating blocks, whereas conventional and real time (Taqman and SYBR green) PCR requires expensive equipment with high maintenance cost. Dedicated and well demarcated space is a must for providing reliable results. This includes separate spaces or rooms for preparing the master mix (clean room), nucleic acid extraction, sample addition, nucleic acid amplification (thermal cycling), and gel electrophoresis for conventional and nested PCRs. Availability of dedicated and physically well separated spaces are needed to prevent contamination of the specimen and reagents which in turn ensures reliable and

reproducible results when NAATs are being used for diagnosis (64). In practice, qPCR (real time PCR) especially TaqMan chemistry based method is less prone to contamination, easier to carry out and gives faster results and hence, has an edge over the other assays (65).

Specific molecular assays for detection of *Orientia tsutsugamushi* have been described. The targets for these molecular assays include the *htrA* (47 kDa periplasmic serine protease), 56 kDa (56 kDa type-specific antigen), *rrs* (16S rRNA), and *groEL* (heat shock protein Hsp60)genes (5,7). The commonest assay used is the 47 kDa. Though the 47kDa qPCR is commonly used, amplification of the 56kDa gene by nested PCR coupled with Sanger sequencing provides information on the prevalent genotypes in a given area (66).

PCR is most often positive in the early phase of illness(till the 10^{th} day) though samples have tested positive even on the 22^{nd} day of illness (67). The LAMP assay, being an isothermal assay is simple, less expensive and can be used in resource limited settings. Targets used are 47kDa & GroEl. The major drawback is that it requires a laborious standardization process (9,65,66) .

Newer molecular assays like the TruNat Scrub T are currently available. It is a chip-based Real Time-Polymerase Chain Reaction (PCR) test for the quantitative detection of scrub typhus bacteria in blood / Serum / Plasma specimens. The target sequence for this assay is *htrA* gene of *Orientia tsutsugamushi*. At the end of the test run, a scrub typhus "detected" (positive) or "not detected" (negative) result is displayed. And in positive cases, CT value is displayed on the screen. The limit of detection was found to be 339 copies/mL. The major disadvantage is that the evaluation of this kit was performed without testing samples from patients with malaria, dengue, enteric fever, sepsis which are differentials for scrub typhus (68). The major advantages of nucleic acid detection tests are that they are faster, sensitive, and specific depending on the primers. They have a lower limit of detection; these tests can detect both viable and non-viable organisms and the yield is high in the first 2 weeks of infection. Drawbacks of all molecular assays are, they are labour intensive, require skilled operators and are performed in batches with a longer turnaround time in actual practice and the chances of contamination are high (51) . Nested PCR involves 2 rounds of PCR, hence, most prone among the PCR assays for contamination. Real time PCR using TaqMan chemistry (primers and probes) provides more rapid results in real- time but 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. This also provides information on the circulating genotype.

Antibody detection (serological assays)

Detection of pathogen specific antibodies in patient's blood may indicate the current or recent infection or a past infection/exposure depending on the type of antibodies detected. Antibody detection in serum, plasma or whole blood is the mainstay of diagnosis in Scrub typhus (69).

Detection of IgM in the patient's serum is considered an indication of a recent infection or an on-going infection, whereas IgG detection indicates past infection to the infectious agent. IFA is the serological reference standard and considered by many "The gold standard" for diagnosis (70). By definition, definitive diagnosis by serological mean should demonstrate rise in titre or sero-conversion (71). Serological assays including IFA can be false negative

early in the illness. This is because IgM antibodies are rarely detected (< 5 days), whereas molecular targets specific to *Orientia tsutsugamushi* can be detected(57). Practical difficulty is having a cut off serological assays as it should be tailored for the specific geographic region and the other AUFIs prevalent in that area which present with similar clinical picture (72). The 56kD antigen is immunodominant and is the most abundant outer surface protein of *O. tsutsugamushi*. All the individuals infected with *O. tsutsugamushi* develop an antibody response against this antigen. However, due to the genotype variations, genotype specific antibody response is developed requiring the knowledge of circulating genotypes to use the appropriate antigen in the test (73). As all the genotypes cannot be incorporated in diagnostic test, region specific need for inclusion of genotypes specific 56-kda antigen in the diagnostic is essential. The different serological assays are listed in the Table 2. The advantage and disadvantage of various serological assays are briefly described in Table 3.

The best performance of the serological assays for scrub typhus is only from the second week of illness. Sensitivity is lowest for Weil Felix test and reaches a maximum of 50%. Specificity has been reported to be low (74), but the authors of this review have consistently observed a specificity above 90% (14). A diagnosis of scrub typhus can be confirmed serologically by demonstrating, a fourfold rise in titre, for which paired samples (acute and convalescent taken at least 2 weeks apart) have to be tested (75). Though this can effectively confirm the diagnosis, is of great epidemiological value, it is always retrospective and of little clinical use. Almost always, the serological diagnosis is performed using the acute specimen. Therefore, determining the cut off value (clinically significant value) becomes important in each geographical areas as re-infections are quite common (57,76). The clinically significant value or titre can be robustly detected by testing known positive samples, samples from healthy individuals, and from individuals with diseases which have clinical features (especially eschar negative) similar to scrub typhus. The interested reader is referred to extensive reviews on this for further guidance (57,70,76).

Assays of the future

IgM capture ELISA

All the commercial IgM ELISA for scrub typhus uses indirect method of antibody detection where the antigen is coated on the solid support followed by the addition of serum and the detection of bound IgM using anti-Human IgM conjugate. This method often suffers from both false positivity and false negativity due the rheumatoid factor and presence of high level of IgG. The issues with the Indirect IgM detection assay can be most effectively resolved by using IgM Antibody Capture (MAC) ELISA and routinely used for the detection of anti-viral IgMs. There is one report of IgM capture ELISA for the detection of IgM against *O. tsutsugamushi* no commercial product yet available. (77). It is expected that a well optimised MAC ELISA for *O. tsutsugamushi* will help early and accurate diagnosis of scrub typhus.

Multiplex PCRs

Multiplexing PCR is done by targeting many genes (47-kDa antigen and the GroEl protein) and is found to be more sensitive(86%) and specific(100%) than the other molecular assays (78).

Multicopy gene vs Single copy gene assay

When compared with 47kDa qPCR, *traD* gene qPCR was able to detect more number of cases. The *traD* gene is also said to be specific to *Orientia tsutsugamushi.* This is a qPCR where multiple alleles of the *traD* (conjugative transfer protein D that encodes for a subunit of type IV secretion system) gene are amplified. Single copy gene amplification yields high cycle threshold (Ct) values. Preliminary evaluation suggested that the copy number of *traD* varies in different strains of *Orientia tsutsugamushi*, ranging from 32 to 1000 copies (56). The single copy qPCRs typically need >10 genome copies/reaction to give a positive signal, whereas amplification was consistently obtained with *traD* for 0.1 genome copies/reaction (79). Further, Prakash JA et al have observed that *traD* qPCR was found to be more sensitive than 47 kDa qPCR for detecting *Orientia tsutsugamushi* in chiggers(80).

As newer species of Orientia are being discovered, a genus specific qPCR like the one described by Jiang et al (2022) could be of use. This 16S rRNA based qPCR, detects a 94 bp region common to the three *Orientia* species (*O. tsutsugamushi*, *Candidatus* O. chiloensis and *Candidatus* O. chuto). Laboratory evaluation confirmed the utility of this assay as it detected all 47 Orientia positive specimens which included 5 *Candidatus* O. chiloensis and one *Candidatus* O. chuto while being negative for the 22 rickettsia species, 11 unrelated bacterial specimens, one human DNA and 4 mouse DNA samples. Further, a field evaluation confirmed its usefulness as it correctly identified 28 cases confirmed by nested PCR(81)

Chemiluminescence immunoassays

Chemiluminescence immunoassay (CLIA) often uses antigen/ antibody coated paramagnetic particles for detection and has higher sensitivity and specificity when compared to ELISA (82,83). Batch testing is not necessary as samples can be tested as they arrive in the lab, that is on demand testing becomes a reality. Further even small concentrations of the biological analyte in the samples can be detected (84). The biggest advantage of CLIA is wider dynamic range which may allow setting more appropriate cut-off for the test (84) . Currently, CLIA has been evaluated and found to be very useful for laboratory diagnosis of HIV, Hepatitis B (85), adenovirus(86), HCV, syphilis (87), *T. gondii* (88), Varicella Zoster (89) and West Nile virus (90) .

Cell based IFA

This has been used effectively in autoimmune disorders(91), and the same technique can be used for infectious diseases like scrub typhus. The need to create chimeric antigen becomes necessary as there are so many genogroups and genotypes. TSA of *Orientia tsutsugamushi* 56 kDa is the major outer membrane protein, major determinant of OTS serotypes (92). Using Baculo virus expression system and transfected *Spodoptera frugiperda* (Sf9), baculovirus expressing the TSA core region were formed. rTSAs are used for IFA in the diagnosis of Scrub typhus. Preparation of OTS infected cells require a BSL3 facility but recombinant antigens can be prepared in a BSL 2 laboratory. Recombinant IFA has also been found to be better than OTS infected cell based IFA and is cost effective (93) The authors are in the process of developing a cell based IFA for scrub typhus.

Fluorescent nanoparticle based lateral flow immunoassay:

Conventional lateral flow assays (LFA), often referred as rapid diagnostic tests (RDTs), use either colloidal gold or coloured polystyrene particles as tracer molecule for the visual readout of the test by naked eye(94,95). Saraswati et al have extensively reviewed the performance characteristics of these point of care tests (POCTs). Their meta-analysis suggests that these tests have a sensitivity that varies from 0.37 – 0.86(mean -66%) and specificity of 0.83-0.97 with a mean specificity of 92% (53). Moreover, visual LFA brings subjectivity in reading the results and also prevent assigning any cut-off as the results are binary i.e. test line observed or no line observed (96). These problems with 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 by replacing colloidal gold with UCNPs the analytical sensitivity of malaria RDT was enhanced by more than 100 times (97).

Assays to detect *Yersinia pestis*, *Bacillus anthracis* spore and *Brucella spp* (98) were evaluated in China and found to have a detection limit 10^5 CFU/ml, developed and evaluated an UCNP based assay for detection of anti-*Trichinella spiralis* IgG antibodies in pig serum. This assay is very promising as it showed very good concordance with gold standard (99). A similar advancement with scrub typus detection could be a game changer.

Next generation sequencing (NGS)

Recently, next generation sequencing (NGS) performed on clinical samples, metagenomic NGS (mNGS) has been used for identifying the etiology when conventional techniques have been unable to unravel the pathogen. NGS on various biological samples has been successfully performed to detect *Orientia tsutsugamushi* (98-103). The summary of the cases for which NGS was used is described in the following paragraph.

NGS detected *Orientia tsutsugamushi* (317 reads) in plasma on day 16 of illness in a 76-year old Chinese patient with fever, kidney injury who was eschar negative. The plasma PCR performed subsequently, was positive (100). A 51 year old female farmer from Guangzhou (an ST endemic area), was diagnosed by NGS on 12th day of fever as ST (377 reads) followed by PCR confirmation, (101). In a patient with fever (no eschar), myalgia and UTI, was identified as scrub typhus by detection of 7 *Orientia tsutsugamushi* reads by NGS on day 13 after serological tests for various bacteria (not scrub typhus) and viruses were negative (102). NGS identified 9 of 13 suspected patients as scrub typhus in Ganzhou, China, one of whom was positive by Weil-Felix test and another by qPCR. *Orientia tsutsugamushi* specific reads ranged from 2-460 (103). NGS on blood sample taken on the 11th day of fever showed 226 reads corresponding to *Orientia tsutsugamushi* in a 51year old botanical garden worker. Scrub typhus qPCR performed subsequently was also positive, confirming the etiology (104). In a patient in Jiangxi, China (a ST endemic area), 6 reads out of 16,422,429 from alveolar lavage fluid and 22 reads of 15,608,629 (blood) were similar to *Orientia tsutsugamushi* (105). Interestingly, IFA or ELISA for scrub typhus was not performed on any of the patients listed here who were diagnosed to have scrub typhus by NGS (100–105). In all these patients NGS was performed in the second week of illness when IFA or ELISA are in all probability going to be positive (14).

Currently, mNGS is not available in the vast majority of hospitals especially those primary and secondary care centres. Hence testing has to be outsourced and reports are not available immediately. Even if mNGS is available, it cannot be performed on demand, takes a minimum of 2-3 days and requires expertise for analysis and interpretation (100–105). The authors of this review feel that in the current situation, mNGS has a limited role in diagnosis as most cases report to centres with very limited resources for diagnosis.

To summarize, the 56 kDa antigen determines the genotype which is directly related to the variation in the 4 variable domains. As genotypes are different across countries and even regions, knowledge of the circulating genotypes becomes essential for designing serological and molecular assays. Therefore, colleagues who are working on scrub typhus diagnostics should be aware of the circulating genotype so that assays which are region specific can be devised.

Conclusion

Scrub typhus has been known to cause severe illnesses in people throughout the world as the pathogen *O. tsutsugamushi* been found to cause infections outside the traditional "Tsutsugamushi triangle". Adding to this concern is the growing number of misdiagnosed or underdiagnosed cases as the symptoms for this illness is vague and non- specific Some of the challenges in diagnosing *O. tsutsugamushi* in laboratories need BSL3 containment, skilled personnel, and specialized equipment. Serology is the mainstay of diagnosis of scrub typhus with IFA the serological reference standard. Intra and inter observer variations (subjectivity) makes it a less useful test in addition to high cost and limited availability of the necessary infrastructure.

Currently, a combination of clinical features, molecular (47q PCR) and serological assays (IgM ELISA) are being used for confirming the diagnosis in suspected cases. In resource poor centers, rapid diagnostic assays combined with clinical features will be most effective. Therefore, we expect that serological assays which are more objective and easier to perform, in resource limited settings, will become available in the future.

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Captions of tables and figures

Table captions

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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 & time consuming Requires trained personnel Requires BSL 3/ABSL3 containment facility Clinically not useful (Median time to detection is 27 days) Requires PCR and IFA for confirmation Low sensitivity (50% at best)	(4,106–109)
PCR (cPCR, nPCR & qPCR)	Positive is confirmative Sensitive and specific: Acute phase Real-time results: qPCR Phylogenetic analysis: cPCR & nPCR LOD (10-50 copies/ reaction)	Requires technical expertise Requires expensive reagents and equipment Contamination issues especially with nPCR Best yield: Acute phase (upto weeks) Specific treatment reduces sensitivity	(51,65,70,110,111)
LAMP & RPA	Isothermal reaction Sensitive and specific depending on the primers Can be performed on various specimens LOD (comparable with nPCR & qPCR)	Requires technical expertise Reagents expensive Extensive validation process required Yield is high only during the first two weeks (acute phase) Specific treatment reduces sensitivity	(51,70,112–115)
IHC & IIP	Can be done in eschar biopsies and other specimens Rapid detection compared to culture methods	Requires technical expertise Expensive, requires fluorescence microscope	(116)

Table 2. Details of assays available for serodiagnosis of scrub typhus.

Parameters	**Weil Felix**(4,51,117)	**ELISA**(4,118,119)	**IFA**(57,120)	**RDT**(82,121–123)
Antibodies detected (predominant)	IgM	IgM/IgG	IgM/IgG & 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	<30mins
Results	Semi-quantitative (titre)	Qualitative (Pos/neg)	Semi-quantitative (titre)	Qualitative (Pos/neg)
Inter and intra observer variation	+	-	+++	+
Antigens used	Heterophile (*Proteus spp*.)	Recombinant 56kDa	Whole organism + host cell antigens	Recombinant 56kDa
Definitive diagnosis (paired sera)	Rise in titre	Sero-conversion	Rise in titre	Seroconversion
Cross reactions	+++	+	++	+
Use in sero-surveillance	+	+++	+	+
Cost effectiveness	++	+++	+	+

***TAT – Includes both time for testing and result reporting **Sensitivity and specificity varies for each assay from a low 50% for Weil Felix to as high as 100% in certain studies for ELISA, IFA and RDTs.*

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	(8,120,124)
ELISA	Objective, economical and technically simpler With automation, large number of samples can be tested Assay can be automated Minimal inter-assay and and intra-assay variation Useful for surveillance studies Can detect lower levels of antibodies compared to IFA	Requires instrumentation: ELISA reader or ELISA workstation Batch testing (single samples cannot be tested) Region wise cut-off values have to be defined ELISA is qualitative Rise in titre difficult to demonstrate	(118,121)
RDTs	Rapid turnaround time and can be used in field settings Simple to perform (point of care testing) Does not require specialized equipment	Chances of error due to prozone phenomenon (since no dilution is performed) False positive results high if not properly validated Indeterminate results due to faint bands Cost per test high (more expensive)	(117,122,123, 125)
Weil Felix	Economical Semi-quantitative Paired sera: Rise in titre demonstrated Specificity: Moderate to good Easy to perform and commonly used in resource limited settings	False positives result due to cross reactivity with other rickettsial organisms, *Proteus* infections particularly UTI Subjective results in inexperience hands Sensitivity low (≈50%)	(4,126)

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	47kDa 56 kDa GroEL 16S rRNA	3-7% 0-96% 66% 45%	100% 100% 100% 87%	(65,127,128)
	Nested PCR	47kDa 56 kDa GroEL 16S rRNA	81-85% 16-100% 90.4% 20.6%	100% 88-100% 100% 100%	(65,67,129,130)
	Real time PCR	47kDa 56 kDa GroEL 16S rRNA	63-81% 65-73% 56.4% 52%	90-100% 100% 96.2% 100%	(65,131,132)
	LAMP	47kDa GroEl	52% 87.5%	94% 100%	(115,133,134)
	RPA	47kDa	86%	100%	(112,113)
Serological tests	IFA	Karp, Kato & Gilliam	78-97%	98-100%	(120,124,135)
	ELISA	56 kDa antigen cocktail	70-100%	87-100%	(119)
	RDTs	Single/Mixture of recombinant 56 kDa proteins	61-100%	74-100%	(122,123,125)
	Weil Felix	*Proteus spp.*antigens (OX-K)	50-60%	95%	(4,126)
Antigen detection	IHC/ IIP	Mouse polyclonal anti *Orientia tsutsugamushi* antibody	65%	100%	(136)

Figures

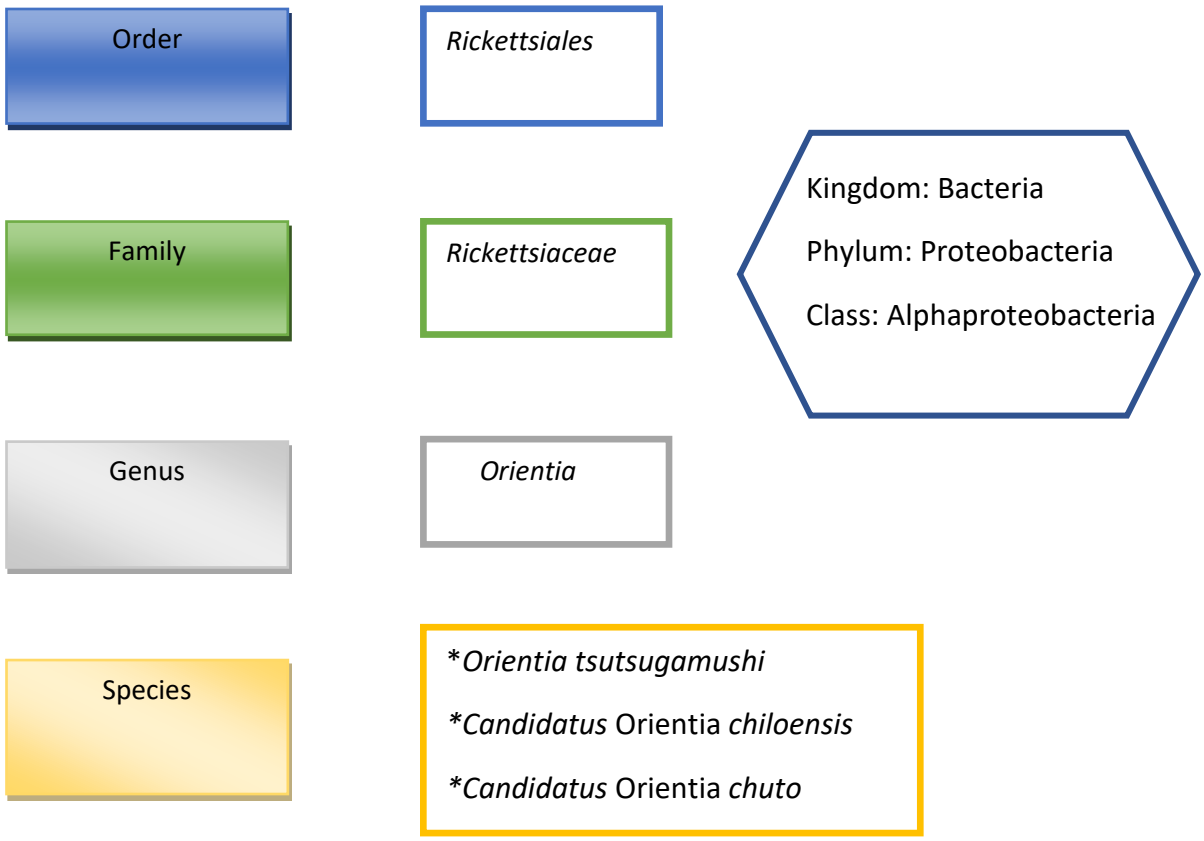

Figure1. Classification of *Orientia tsutsugamushi*

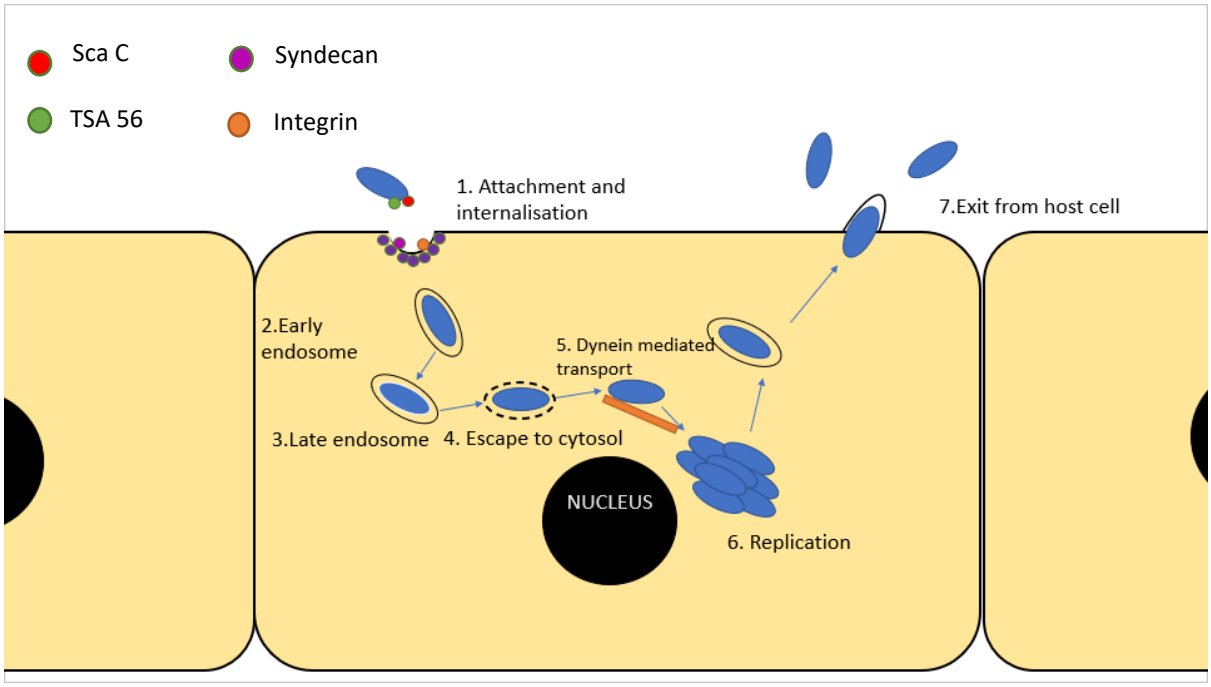

Fig. 2: Pathogenesis of scrub typhus