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Review Article

Scrub Typhus: A Threat to 'Tsutsugamushi Triangle'

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Abstract

Scrub typhus, caused by *Orientia tsutsugamushi*, is an important vector-borne disease that has been classified a neglected tropical zoonosis. The ecology of the disease is complex and less understood thus has more public health implication than other zoonotic diseases. About a million cases of scrub typhus are reported worldwide annually and the disease is associated with high mortality. It is endemic in geographically confined area of the Asia-Pacific termed as the Tsutsugamushi triangle', which is bound by Siberia and Kamchatka peninsula to the north, Australia to the south, Japan to the east and Afghanistan and India to its west, covering Northern Australia, South and Southeast Asia, the Indian subcontinent and Pacific Ocean. There has been a resurgence of scrub typhus across India in the recent years and the disease has re-emerged as a major cause of acute undifferentiated febrile illnesses (AUFI) in humans with high morbidity and mortality. Thus better understanding of scrub typhus epidemiology is needed to put in place appropriate public health interventions to reduce the burden of the disease globally. This comprehensive review provides summary of work, investigation of this pathogen in vectors and updates for understanding the complexity of scrub typhus

Keywords: Scrub Typhus; Orientia Tsutsugamushi; Tsutsugamushi Triangle; Vector-Borne Zoonosis

Introduction

Scrub typhus (ST) is a mite-borne zoonotic disease caused by an obligate, intracellular bacterium, *Orientia tsutsugamushi* which produces a life-threatening febrile condition in humans with high case fatality rate. With many synonyms like Tsutsugamushi disease, Mite-Borne Typhus, Tropical Typhus and Coastal fever, Scrub typhus has described as one of the most underreported diseases of the world-by-World Health Organization (WHO) [1]. The disease is endemic to Asia-Pacific region, often called as the "tsutsugamushi triangle", but is now expanding its area of coverage to all around the world because of industrialization and reported cases are near about one million every year around the globe [2]. The bacterium spreads through the bite of larval stage of infected *Leptotrombidium* mites which maintain the organisms through transstadial and transovarial transmission [3]. The disease is characterized by sudden increase in body temperature, maculopapular rashes, myalgia, bradycardia and headache [4]. As it is a growing concern for the entire world, immediate attention is required for implementation of effective preventive and control measures.

Etiology

The causative pathogen of Scrub Typhus is *Orientia tsutsugamushi*, a gram negative, obligate intracellular rickettsial bacterium belonging to the family Rickettsiaceae and order Rickettsiales, transmitted to warm-blooded animals by the bite of larva of chigger mite *Leptotrombidium* of the family Trombiculidae [5]. More than 30 serotypes of *Orientia tsutsugamushi* having high antigenic variation in outer membrane protein have been reported so far from the endemic areas of Tsutsugamushi triangle. Out of these serotypes, most common are *Karp*, *Kato*, *Gilliam*, *Boryong* and *Kawasaki* [6]. Now scrub typhus is not only confined to its endemic areas but spreading outside tsutsugamushi triangle, spilling to the Middle East, South America, U.A.E (Dubai). In India, most of the studies reported the high predominance of Karp-like strain in the north (Himachal Pradesh) and south (Kerala, Vellore, Andhra Pradesh) of the country followed by Kawasaki-like and the Kato-like serotype that are prevalent in northern, southern and north-eastern part of the India. *O. tsutsugamushi* clades isolated from North-India (Punjab,

Haryana, Himachal Pradesh, and Chandigarh) have been found to be closely clustered with Boryong prototype [7]. Variations in serotypes of different geographical provinces suggest the need for a deeper and more thorough study to get a better perception of its distribution

Epidemiology of scrub typhus

Until the beginning of the twenty-first century, Scrub Typhus has been described as a neglected tropical zoonosis due to insufficient data for epidemiological studies [8]. The disease is prevalent in South and East Asia and parts of the Pacific Rim. Almost one billion people in the endemic areas are at risk for acquiring it and one million new infections are reported annually. Scrub typhus became known to the Western world during World War II when it caused tens of thousands of cases in Allied and Japanese troops [9]. The disease was heavily reported in Vietnamese army personnel during Vietnam War in the 1960s. The disease is highly endemic to "tsutsugamushi triangle" covering more than 8 million square kilometers of area from far eastern Russia in the north, to Pakistan in the west, Australia in the south, and Japan in the east [10]. In the Asia Pacific, the cases are primarily found in the southwest, southeast coastal and eastern regions of China. The countries located in "tsutsugamushi triangle''are Japan, Indonesia, Malaysia, Philippines, Pakistan, Afghanistan, Far East Russia and Australia.

The natural history of scrub typhus is influenced more by the life-cycle and biology of the vector and its interaction with hosts and the environment rather than behavioral and demographic factors [11]. Major elements that play an important role as risk factors include the presence of scrub vegetation, woodpiles, and the susceptible cattle around the residences, living in the vicinity of the water body, cooking outside the home, domesticating pets and cattle etc [12]. Susceptible age-groups seem to vary a lot. In China, 60-69-year-old people were reported to acquire the disease during June-July but maximum cases were from 51-75 years age group [13]. In Japan and South Korea, most cases are recorded in October and November while in South Korea, Scrub typhus accounts for 27.7-51% of total acute febrile illness cases affecting more females than males [14].

Similarly, Japan and Taiwan are also following the trend with more reports in the female to male ratio among the age group of 50-60 years [15]. In India, scrub typhus is still an under-diagnosed disease with cases reported from all over the country especially in rural areas between August to November [16]. Cases are now emerging outside the traditional endemic areas possibly because of introduction of new species of *Orientia*, or new vectors which are yet to be identified [17].

Transmission

Scrub typhus is transmitted by bites of larvae of trombiculid mites (chiggers) of the genus *Leptotrombidium. The ticks transmit the organism* vertically by transovarian (transmission through eggs) and trans-stadial (passage from mite larva-nymph-adult) route and there is no evidence of horizontal transmission of scrub typhus so far [18]. The following species of *Leptospira* are involved in the transmission in different countries. *L. deliense* is the important species in South East Asia and southern China, whereas in Japan and Korea main vectors are *L. scutellare*, *L. pallidum* and *L. akamushi* while in Thailand involved species are *L. chiangraiensis*, *L. imphalum*, *L. deliense* and *L. scutellare* [19].

Pathogenesis and Clinical Signs

The trombiculid mite harvests the bacteria (*Orientia tsutsugamushi)* in its salivary gland where infection and activation of the endothelial cells occur that in turn assist in scrub typhus morbid physiology. Subsequently, enhancement of the infection to several organs including heart, kidney, skin, pancreas and brain takes place [20]. Scrub typhus pathophysiology is still not clear but immune-mediated processes as well as direct bacterial damage seem to be responsible for the local or disseminated pathological mechanism of the organism [21]. The organism targets wide range of host cells including dendritic cells and activated monocyte cells for completing its replicative cycles and spread in the body through lymphatic system [22].

Clinical sign in human diseases can range from mild (asymptomatic) flu-like (fever, headache, myalgia, nausea, vomiting) to severe such as meningitis, intravascular complications, severe pneumonitis/peritonitis, altered sensorium and/or cardiac distress [23]. Along with, fever, breathlessness, cough, headache, nausea/vomiting, and altered sensorium are also accompanying observations, moreover, a hemophagocytosis syndrome (HPS) has been associated with scrub typhus [24]. The HPS can be diagnosed in phagocytosed blood cells in bone marrow aspirates with cytologic findings of histiocytes. It is important to relate the HPS with scrub typhus, as the prognosis can be poor in untreated cases [25]. It is noteworthy that both the spotted fever group rickettsia and scrub typhus can include an inoculation eschar at the bite site, so differential diagnosis should carried out at different levels [26].

Diagnosis of scrub typhus

Scrub typhus fever shows similarity with other febrile illnesses such as dengue, typhoid, leptospirosis, and murine typhus thus making the diagnosis confusing. The presence of eschar over the body in patients is the characteristic lesion which aids in the diagnosis of scrub typhus while in absence of pathognomonic eschars, laboratory tests confirmed the infection [27]. For the accurate diagnosis of scrub typhus presumptive as well as definitive tests should be performed. Laboratory-based methods are more reliable and precise and can be direct and indirect. In the direct method of diagnosis isolation and culturing of bacteria in different cell lines like L929 (normal fibroblast cell line of the mouse), HeLa (cancer cell line), BHK21 (baby hamster kidney fibroblast cell lines), Vero lineage (isolated from kidney epithelial cells extracted from an African green monkey) is performed. Further their DNA-based diagnosis by PCR using different genetic markers is carried out. Various serological assays such as Weil-Felix test, immunofluorescence assay (IFA), enzyme-linked immunosorbent assay (ELISA) and rapid diagnostic assays are indirect methods of diagnosis [28].

Method of diagnosis of scrub typhus

Indirect methods

- Weil Felix Reaction
- Immunofluorescence Assay
- Immunochromatic Test
- Indirect Immunperoxidase Assay
- ELISA

Direct methods

- Bacterial isolation and culturing
- Polymerase chain reaction

Indirect methods of diagnosis

- **Weil-Felix Test**: This test is a non specific agglutination test based on cross reactivity between certain non-motile Proteus serotypes with certain Rickettsial species. The test serum gets agglutinated due to presence of Weil-Felix anibodies. OXK reactive agglutinins appear 10-14 days after onset of scrub typhus and decline subsequently with time. Due to the pervasiveness of the *Proteus* species, lower serum titers below 1:160 are not considered significant. However this serum titer level doesn't achieved frequently, so only preliminary diagnosis can be made by a four fold increase in titer during infection.. Chances of false-positive results are very high in the case of *Proteus* infection of the urinary tract, leptospirosis, and relapsing fever. A negative Weil-Felix test results may not exclude scrub typhus and requires further confirmation. This test has very less specificity and sensitivity due to the use of non-rickettsial antigen for agglutination. Despite low specificity, in set-ups with inadequate diagnostic facilities, Weil-Felix test could be used as a primary screening method for qualitative estimation with clinical co-relation for better patients' management [29].
- **Immunofluorescence Assay (IFA):** Uses fluorescein linked anti-human reporter antibody to detect the presence of scrub typhus-specific antibodies in the serum sample. The most frequently used antigens are Karp, Kato and Gilliam's in indirect IFA for the diagnosis of scrub typhus [30]. Indirect IFA considered as a gold standard test for the detection of scrub typhus due to its higher sensitivity and specificity. However other techniques like ELISA also have been reported with similar sensitivity and specificity with lower cost [31].
- **Immuno Chromatographic Test (ICT):** ICT has been considered a point of care diagnosis system to detect scrub typhus because of its short rapid response time (10-15 min) without requirement of sophisticated instrument facility. Its sensitivity and specificity is reported to be similar to the other standard methods used for its detection. The sensitivity of ICT has been shown to increase by 20% when used along with DNA-based loop-mediated isothermal PCR assay (LAMP). To achieve still higher sensitivity and accuracy, a surface-enhanced Raman scattering-based lateral flow assay (SERS-LFA) has been developed. So, ICT assay has a promising future in the diagnosis of scrub typhus due to its excellent sensitivity, specificity, field-deployment. Further, it can be used as a diagnostic method especially in rural areas where IFA facility is not available [32].
- **Indirect Immunoperoxidase Assay (IPA):** Immunoperoxidase assay is similar to IFA with the only substitution of fluorescein with a peroxidase enzyme that reduces its cost due to the elimination of expensive equipment such as a fluorescent microscope. Indirect IPA was evaluated for the diagnosis of scrub typhus using a smear of infected mice spleen with strains of *O. tsutsugamushi* and found to be a better alternative for IFA with the advantage of permanent preparations for re-examination and ability to observe infected and uninfected cells. Another advantage of IPA over IFA is that any serotype can be used as an antigen and either IgG or IgM can be measured individually. IPA could be used as an alternative test for IFA in areas where sophisticated instrument facilities

are not available. However, its subjective readings make it inferior to other serological methods such as ELISA [33].

- **Enzyme Linked Immunosorbent Assay [IgG and IgM]:** ELISA is one of the best serological methods for the detection of scrub typhus due to its better sensitivity and specificity than the present gold standard method, IFA [34]. Even though IgM-based immunofluorescence assay is a gold standard test for the diagnosis of scrub typhus, it has some drawbacks like its higher cost, the need for trained personal, and the requirement of a fluorescent microscope [35]. Most of the ELISA based methods target IgM and IgG antibodies in serum samples for the detection of *Orientia tsutsugamushi*. IgM and IgG antibodies titer can be observed after the 1st and 2nd week of infection, respectively. That's why, IgM-based ELISA methods are better for early detection and can be differentiate nascent infection with the past one. A modified Dot-ELISA using a combination of recombinant proteins used for diagnosis of scrub typhus. So, IgM-based ELISA may become a better alternate and possibly viable option for resource-limited endemic countries [36]. The main drawback of the immuno-based methods is the lack of standardization and the choice of reference test to compare the ELISA, bringing into question the validity of cut-offs used in diagnostic accuracy studies and observational studies. To improve the accuracy of the diagnostic methods, studies need to be done in different geographical locations to determine the region-specific cut-offs for scrub typhus [37].

Direct methods of diagnosis

Bacterial Isolation and Cultur in

Direct methods of diagnosis involve bacterial isolation and culturing in chick embryos or established cell lines and their detection using molecular assays such as PCR. Several genetic markers such as *56 kDatsa*, *47 kDaHtrA*, *GroEL*, and *16s rRNA* genes are targeted in PCR. These methods can detect infection in early stages with higher sensitivity and specificity. However, these methods have several drawbacks like the requirement of biosafety level (BSL) 3 facilities for culturing of *Orientia tsutsugamushi* and higher genetic diversity among serotypes of scrub typhus [38].

The cultural demonstration of the organism in conventional hemoculture performed before molecular testing could increase the sensitivity of *O. tsutsugamushi* molecular diagnosis. Dittrich., *et al.* [39] conducted a study in Laos using hemocultures, one pair of which was aerobically incubated at 35°C-37°C for 7 days and subsequently subjected for qPCR using serial dilutions of pGEM-T Vector Systems. If the employed EDTA buffy coat qPCR had given results within the 7-day hemoculture incubation period, the respective bottles were sampled at subsequent times in order to have PCR positivity estimation.

Isolation of the organisms can be done in cultured mouse embryonic fibroblast cells (L929) after which the growth can be measured using an *O. tsutsugamushi*-specific qPCR assay. Mouse fibroblast line can be cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) without antibiotic, unless stated otherwise. The culturing of *O. tsutsugamushi* (strain UT-76, a Karp-like strain from Thailand) was propagated in T25 cell culture flasks containing the confluent monolayer of L929 cells which after 7 days were sub-cultured. Thereafter the bacteria were lysed and subjected for qPCR targeting the 47 kDa gene or cfd gene for *O. tsutsugamushi* or L929 cells, respectively [40].

Besides, to develop a monolayer of cells, the confluent L-929 cells on harvested by trypsinization can be resuspended in M199 medium and exposed to 3,000-rad irradiation in 60 ^{0}C gamma irradiator and these irradiated cells can then be used for monolayer development [41].

Polymerase Chain Reaction

Historically, the gold standard diagnosis of scrub typhus was based on serology, but the added value of DNA-based pathogen detection in the early bacterial dissemination phase of disease has resulted in a combination of "polymerase chain reaction (PCR) plus serology" for the diagnosis of most rickettsial diseases. PCR assays now represent a central pillar in scrub typhus diagnosis because of its high diagnostic accuracies.

The PCR requires genetic markers which makes the detection more sensitive and specific. For the detection of *O. tsutsugamushi,* markers like *56 kDa tsa, GroEL, 16s RNA* and *47 kDa HtrA* are usually used [42,43]. Mostly *56 kDa tsa* gene is preferred for strain characterization of the organism. But a conserved region of *GroEL* gene possesses better sensitivity that helps in overcoming the limitation of sensitivity and specificity hindrance due to human blood DNA contamination in the concerned samples as the bacteria prefer intracellular habitat [44]. Apart from these, real time PCR is more preferrable because of its higher sensitivity and specificity. Many researchers have targeted different genes to detect scrub typhus. Hydrolysis probes, 16S rRNA gene whereas 60-kDa heat shock *Gro-*

EL gene using SYBR green had been detected by (Jiang., *et al*. [45], Sonthayanon., *et al.* [46]. In fact, development of a noble multiplex real-time PCR targeting multiple genes like 47-kDa antigen encoding gene and GroEL protein encoding gene with human interferon beta (IFN-β) proved to be better for diagnosis of scrub typhus with 86% sensitivity and 100% specificity [47].

Besides, LAMP is also very much useful in the detection of the bacterium. It works on isothermal amplification of DNA using polymerase and primer sets. Target of 60 kDa heat shock protein of *Orientia tsutsugamushi* encoding *groEL* gene is helpful in detecting acute form of scrub typhus [48].

Though the validity of the tests rely on scrub typhus infection criteria parameters, viz., positive cell culture isolation, four-fold increased IgM titers in paired sera (admission IgM should be more than 1:12800) and more importantly positive PCR assays atleast in two out of three using target genes of *56 kDa tsa*, *47 kDa HtrA* and *groEL* [49].

Conclusion

This review tries to highlight the epidemiology and available methods of diagnosis with challenges in the diagnosis of scrub typhus while focusing on the future perspective of scrub typhus diagnosis. Till now IFA has been considered as the gold standard for diagnosing Scrub Typhus but DNA based studies are the keys of early-stage diagnosis of scrub typhus. Practically, these assays are more rapid and specific to those of Weil-Felix, ELISA, IFA etc. tests. These methods are still in experimental stage and require detailed studies. As the biosensor-based disease diagnosis have higher sensitivity and specificity, these are getting more highlighted and becoming the future of the disease diagnosis. We are continuously striving towards developing a superior care-diagnosis system to conclude a hasty and precise diagnosis of any disease. Therefore, focus should be made on detection techniques of scrub typhus with more advantages and rather no drawbacks including easier and rapid disease detection.

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