Review Article: Unresolved Problems Related to Scrub Typhus: A Seriously Neglected Life-Threatening Disease

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Ecology and epidemiology. Scrub typhus is a life-threatening infectious disease that presents as an acute undifferentiated febrile illness. Its agent, *Orientia tsutsugamushi*, is an obligately intracytosolic bacterium that is transmitted by feeding larval trombiculid mites, which are the reservoir of the agent and the only life stage that feeds on a vertebrate host.[1] Nymphal and adult trombiculid mites live in the soil and feed on the eggs of insects. Mites maintain the organisms by transovarian transmission as well as transtadial transmission through the mite's lifecycle.[2,3] Although mites transmit *O. tsutsugamushi* to vertebrate hosts such as rodents, only a small proportion of uninfected mites acquire *Orientia* during feeding on infected animals, and the ingested *Orientia* do not establish disseminated infection in the mites and are not transmitted transovarially to the next generation.[4–7] It seems that chigger cofeeding on rodents is more relevant for effective mouse-to-mite transmission of *Orientia* than feeding on rickettsemic hosts.[5,8] Thus, the true role of rodents as reservoirs requires additional investigation, because there may be marked genetic variability in chiggers with respect to the ability to acquire rickettsiae by feeding. Also, rodents may be better considered as dead-end hosts just as humans are rather than a reservoir. However, the evolutionary selection of tremendous antigenic diversity of the immunodominant major 56 kDa surface protein presents an enigma if immune variation plays no role in survival of *O. tsutsugamushi*.[9] The potential immunomodulatory effects of the saliva of larval mites on the pathogenesis of and immunity to infection with *O. tsutsugamushi* remain undetermined.

One million cases of scrub typhus occur each year with an estimated 10% case fatality rate unless treated appropriately, very likely resulting in more deaths than dengue.[10,11] Currently, scrub typhus has predominantly been reported from an area extending from the Russian Far East and Korea in the north to northern Australia in the south and Afghanistan in the west, and it includes islands of the western Pacific and Indian Oceans, including Japan, Taiwan, Philippines, Papua New Guinea, Indonesia, and Sri Lanka.[12,13] This geographical range may be an underrepresentation, because case reports have been published from Africa and South America. The recent isolation of a novel species *O. chuto* acquired by a patient in Dubai, the detection of another divergent *Orientia* transmitted to a patient in Chile, and serologic diagnoses of scrub typhus acquired in Africa indicate that a wider geographic distribution and genetic diversity of the genus should be investigated.[14–17] The burden of disease in rural areas of Asia is large, with studies showing scrub typhus causing up to 20% of febrile hospital admissions,[18–20] an incidence of infections of greater than 3% of the population monthly,[21] seroprevalence over 50% of the population, despite a significant annual rate of reversion to seronegativity of 50% of cases,[22] and a seroconversion rate of 484 per 1,000 person-years.[23]

In 1999, the World Health Organization (WHO) stated, "Scrub typhus is probably one of the most underdiagnosed and underreported febrile illnesses requiring hospitalization in the region."[24] This opinion remains valid today and could justifiably be adjusted to scrub typhus is probably the single most prevalent, under-recognized, neglected, and severe but easily treatable disease in the world. This ancient disease is currently undergoing increased awareness both because of re-emergence and rising incidence in previously unrecognized areas and improved diagnostic testing. It is apparent that scrub typhus has been recognized to occur frequently now in places where the illness was nearly forgotten, including India, Sri Lanka, the Maldives, and Micronesia.[25–28] However, scrub typhus has also emerged in regions north of the Yangtse River in China, where it was not known previously.[29–33] The emergence of scrub typhus should also take into consideration the expansion of farmlands that have generated ideal habitats for trombiculid mites. The potential relationship with global climate change is unclear. Greater recognition in some countries, such as Thailand, Laos, Taiwan, and Japan, may reflect increased medical investigations and application of new diagnostic methods.[34–37] The critical unresolved issues regarding epidemiology emphasize the need to determine the reasons for emergence and re-emergence and the true incidence of this neglected disease, for which calculated days of disability-adjusted years of life lost (DALYs) have not been determined and to which appropriate attention has not been paid (Table 1).

Clinical manifestations. Scrub typhus ranges from a mild to a fatal illness. The early clinical manifestations are an eschar, representing localized cutaneous necrosis at the site of mite feeding (which is not always present), and regional lymphadenopathy followed subsequently by fever, headache, myalgia, generalized lymphadenopathy, cough, gastrointestinal symptoms, transient hearing loss, and rash.[38,39] Progression of severe scrub typhus may manifest as acute respiratory distress, meningoencephalitis, gastrointestinal bleeding, acute renal failure, hypotensive shock, and coagulopathy.[40–42]

Unresolved clinical issues include the reason for the wide range of occurrence of eschars (7–97%), the reason for rash and severity of illness, and the need for characterization and determination of the mechanisms of the coagulopathy, hemorrhages, interstitial pneumonia, and meningoencephalitis.[43–45] There is currently no unified approach to assess and stratify

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TABLE 1

Unresolved issues related to ecology and epidemiology

Determination of the true impact of scrub typhus on public health
Determination of whether there are other important factors for
  maintenance of *O. tsutsugamushi* in nature other than vertical
  transmission in mites that explain the extent of antigenic diversity
Elucidation of the basis for the periodic re-emergence of scrub typhus
Discovery of *Orientia* species other than *O. tsutsugamushi* and their
  actual geographic distribution

disease severity in scrub typhus. A clinically based score or algorithm including validated surrogate markers of disease would support host–pathogen and pathophysiological investigations. The geographic distributions of the currently over 70 known strains of *O. tsutsugamushi* and the tremendous range of disease severity and mortality (including, in part, data from the pre-antibiotic era) of scrub typhus have led to the unproven hypothesis that *O. tsutsugamushi* strains may vary in human virulence.[33,46,47] Strain virulence in experimental animals does not correlate sufficiently with strain virulence for humans to be used as a yardstick of pathogenicity.[48,49] An important unresolved issue is the identification of bacterial virulence mechanisms and host-determined mechanisms that affect disease severity (Table 2).

Diagnosis and diagnostics. There is an urgent need for alternative diagnostic methods for scrub typhus. The current gold standard reference diagnostic method—the indirect immunofluorescence assay (IFA)—is imperfect, because it is often retrospective and requires a level of technical expertise and equipment that may not be available in rural areas. In most studies, the description of the IFA methodology and rationale for seropositivity criteria are insufficient, an arbitrarily defined cutoff titer is often used rather than a dynamic rise in the titers of paired samples, and positivity cutoff titers have varied by country and purpose of the IFA test.[50] This variation limits the comparability of seroprevalence rates among studies and more critically, raises questions about the appropriateness of the cutoff values for a diagnostic IFA result.[51]

With increasing recognition of the heterogeneity of *O. tsutsugamushi* strains across Asia, current investigations should be directed to identification of suitable gene and protein targets containing conserved areas with broad but *Orientia*-specific antigenic reactivity for development of both diagnostics and a vaccine. The comparisons of diagnostic accuracies and performance characteristics of assays for acute diagnosis of scrub typhus infection have been summarized previously.[52]

In a polymerase chain reaction (PCR) diagnostic study in Korea, *O. tsutsugamushi* DNA was detected in eschars from six of seven patients who did not yet have antibodies

TABLE 2

Unresolved issues related to the pathogenesis of clinical manifestations

Determination of the reasons for the wide variation in incidence
  of eschars, rash, and hemorrhages
Elucidation of the pathogenesis of acute respiratory failure and central
  nervous system complications
Characterization of the coagulation abnormalities in scrub typhus
Determination of virulence mechanisms of *O. tsutsugamushi*,
  host-mediated pathogenic mechanisms, and mechanisms
  of tissue injury
Development of a disease severity score based on clinical features
  and pathophysiological markers

against *Orientia* by IFA but had eschars suggestive of scrub typhus.[50,53] A study in Thailand showed that 3 (15%) of 20 patients with fever had detectable *O. tsutsugamushi* DNA by PCR in blood, despite the absence of antibodies.[54] These findings could reflect potential benefits of the PCR assay in detecting *Orientia* DNA before antibody responses occur and a diagnostic advantage in endemic areas with high background levels of antibody in the population. However, the high resource costs and training required for these PCR assays make them impractical in many areas of scrub typhus endemicity. That fact aside, it remains unclear what the most appropriate specimen is for study; PCR of eschar biopsy yields more sensitive results than blood, and *Orientia* DNA can remain in the unperfused part of the necrotic eschar even after the initiation of antimicrobial treatment.[50] However, in a setting where eschars are present in as few as 7% of patients,[43] eschar-based tests would diagnose no more than 7% of cases of scrub typhus. The use of buffy coat sample specimens could improve sensitivity compared with whole blood if the organisms are associated with circulating monocytes or detached endothelial cells,[55] but the use of blood-based assays is limited to the time window of rickettsemia; also, DNA-based assays are only as good as the samples on which are tested. An approach for amplification of bacteria in samples with very low copy numbers is needed. It also remains unclear what the optimal PCR gene target is for diagnosing scrub typhus. A nested PCR assay targeting the 56 kDa gene is highly specific,[56,57] but sequence variability of this gene may affect primer annealing and, therefore, test sensitivity. A target gene, enabling specific but sensitive detection as well as sufficiently broad coverage of genotypes of *O. tsutsugamushi*, is desirable. For example, a 16S rDNA-based *Orientia*-specific primer set may show a broader detection spectrum than an assay based on a more variable species-specific target, such as the 56 kDa gene. Current data on the duration of the rickettsemic period (when PCR-based tests would detect bacterial DNA in peripheral blood) and clinical identification of febrile patients within this phase suggest that a sensitive antigen- or DNA-based diagnostic method could prove beneficial. Because of the rural epidemiology of scrub typhus and delayed presentation, it seems unlikely that such a test would replace serology completely, but (similar to dengue) combined antigen or DNA and antibody detection testing could provide strong diagnostic advantages (Table 3).[58] Hence, diagnostic development efforts should focus on identification of both pathogen- and antibody-based tests. New antigen capture assays should improve diagnostic accuracy in the early phase of infection, whereas serological improvements should aim to eliminate the subjectivity of reading IFA slides (e.g., using standardized scanner-based platforms), reduce cross-reactivities (i.e., a panel of recombinant antigens instead of whole-cell antigens), and enable reliable differentiation between acute and endemic positivity criteria.

TABLE 3

Unresolved issues related to diagnosis and prognosis

Development of a simple, affordable point-of-care diagnostic
  approach (preferably a combined rapid test that detects antigen
  or DNA and antibody for the diagnosis of scrub typhus in the early
  or mid-course (analogous to the NS1/IgM algorithm in dengue)
Detection of a set of markers that are useful early in the disease
  course to assess diagnostic severity and prediction of progression

For comparisons and evaluation of new diagnostic methods, a robust reference set of criteria covering both antibody- and bacterial DNA-based detection should be used that defines scrub typhus with a high level of confidence. Recently, a panel of scrub typhus infection criteria was proposed for diagnostic accuracy evaluations, in which one or more of the following criteria had to be fulfilled: (1) a cell culture isolate, (2) a single admission immunoglobulin M (IgM) titer ≥ 1:12,800 using the gold standard indirect IFA, (3) a fourfold increase in IgM IFA titer, and/or (4) detection of *O. tsutsugamushi* DNA in at least two of three different target PCR assays.[58]

Mouse models. The majority of research on animal models of scrub typhus has been conducted on various strains of mice infected through intraperitoneal inoculation.[59,60] Groves and Osterman[60] used approximately 40 cross-bred, inbred, and outbred mouse strains to study *Orientia* infection. After intraperitoneal infection, all combinations of *Orientia* strains and mouse strains resulted in illness of mice, but none reproducibly developed pathology resembling human scrub typhus. Kundin and others[59] inoculated albino Swiss mice by intracranial, intramuscular, intraperitoneal, and subcutaneous routes, resulting in route-specific *Orientia* distribution and lesions that did not represent human scrub typhus-like pathology.

Intraperitoneal inoculation of *O. tsutsugamushi* into mice results in intense infection of peritoneal mesothelial cells and macrophages and *Orientia* peritonitis with limited systemic spread late in the course.[59,60] Subcutaneous inoculation, which differs from intradermal inoculation by a feeding chigger, results in chronic persistent infection. Intravenous inoculation of inbred C57BL/6 mice results in disseminated endothelial infection and vascular inflammatory and immune responses in lungs, brain, and other organs that represent the pathology of fatal human scrub typhus (Shelite TR, Walker DH, unpublished data). Severity of disease, including lethality, is dose-, *Orientia* strain-, and mouse strain-dependent. Intradermal inoculation of C57BL/6 inbred mice does not cause eschar formation but results in disseminated infection targeting endothelium and mononuclear phagocytes and histopathologic lesions resembling human scrub typhus (Mendell N and others, unpublished data). Validated infection models using inbred mice will allow studies incorporating adoptive transfer of selected immune cells, such as CD4+ or CD8+ T cells. Valid models in C57Bl/6 mice open the opportunity to study genes involved in the mechanisms of immunity and pathogenesis by the use of gene knockout mice. The ideal model would use chigger transmission of a predictably lethal or sublethal dose of *O. tsutsugamushi* into C57Bl/6 and other inbred strains of mice with differences in susceptibility to severe disease. The variations in levels of susceptibility reflected in bacterial loads, dissemination to specific organs, and host responses of outbred mice limit their suitability for specific immunological mechanistic investigations of the immune response.[61]

Non-human primates. Clinical, pathologic, and immunologic responses to experimental scrub typhus infections have been studied in silvered leaf monkeys (*Presbytis cristatus*) and cynomolgus monkeys (*Macaca fascicularis*) indigenous to the endemic area for this disease; the latter was found to be the better model for the study of this disease, mainly because of the difficulty of maintaining silvered leaf monkeys in captivity.[62–65]

In a detailed study, cynomolgus monkeys evaluated for cellular immune responses after intradermal infection with Karp strain of *O. tsutsugamushi* developed eschars, lymphadenopathy, rickettsemia, and elevated body temperatures. The development of IgM antibody titers was followed by IgG antibodies and cell-mediated immune responses shown by lymphocyte proliferation and interferon-γ (IFN-γ) production by *O. tsutsugamushi* antigen-stimulated peripheral blood mononuclear leukocytes. When challenged 6 years after the initial infection, clinical signs and cellular responses were indistinguishable from the signs and responses of naive animals, but an anamnestic IgG antibody response was observed. However, if challenged at 1 year after primary infection, only a localized cutaneous lesion developed. The majority of animals infected previously had persisting lymphocyte responses to antigenic stimulation, suggesting long-term immunologic memory that was not protective against rechallenge.[64] In a recent study, cynomolgus macaques infected intradermally with *O. tsutsugamushi* Karp strain developed eschars and regional lymphadenopathy followed by systemic lymphadenopathy, with pathological features of the eschar closely resembling human scrub typhus. *O. tsutsugamushi* was detected in mononuclear leukocytes in eschars and lymph nodes, and *O. tsutsugamushi*-specific IgM and IgG antibodies and cell-mediated immune responses were observed in all infected non-human primates (Paris DH, unpublished data).[66] Very limited data exist on rhesus monkeys experimentally infected with *O. tsutsugamushi*. The monkeys develop skin ulceration at the site of chigger transmission or artificial intradermal inoculation (subcutaneous inoculation does not produce ulceration) accompanied by regional lymphadenopathy. Histopathologic lesions are characterized as perivascular collections of lymphocytes and macrophages, with multifocal necrosis and white blood cells marginated to the endothelial lining of the microcirculation (Paris DH, unpublished data). It has been shown that experimental scrub typhus disease in rhesus macaques in the terminal stages is pathologically similar to the terminal stages of the disease in humans.[67] To date, no lethal scrub typhus disease model has been developed for rhesus macaques. The inoculation dosages, susceptibility of strains, and infection routes need to be further investigated in view of evaluating the optimal non-human primate model with signs and lesions of sufficient severity and similarity to human signs; this investigation will enable the development of a well-validated non-human primate model of scrub typhus useful for the evaluation of future candidate vaccines.

Pathogenesis. *Orientia* inoculated in chigger saliva infects mainly dendritic cells and macrophages in the dermis underlying the eschar at the site of inoculation.[68] The early development of lymphadenopathy in the regional drainage of the eschar suggests lymphogenous spread.[68,69] Subsequently, hematogenously disseminated infection involves predominantly endothelial cells and to a lesser degree, macrophages, both of which release soluble cell-specific adhesion molecules, suggesting that these cells play key roles in systemic inflammation.[70]

Attachment and entry of obligately intracellular *O. tsutsugamushi* into host cells by a clathrin-dependent endosomal pathway involve the major 56 kDa surface protein, an autotransporter (ScaC), a 47 kDa surface protein, host fibronectin, and integrin-a5b1 and syndecan-4 host cell receptors. The cell biology of this intracytoplasmic pathogen has been reviewed recently by Ge and Rikihisa.[71]

Key unresolved issues regarding the pathophysiology of scrub typhus include identification of the mechanisms of cell

injury, such as potentially direct *Orientia*-mediated intracellular events, generation of reactive radicals by host cells, pathologic levels of cytokines, and cytotoxic immune cells, and actual pathophysiological effects, such as potentially increased vascular permeability leading to edema in vital locations, which occurs in rickettsial infections. Another unresolved topic is the prevalence and pathologic effects of persistent *Orientia* infection.[72,73]

Persistent infection by *Orientia* was first described in experimentally infected mice, and bacteria could be detected by inoculation of blood or homogenized tissue of previously infected mice for up to 610 days after the infection.[74,75] Smadel and others[72] showed that, 2 years after scrub typhus, *O. tsutsugamushi* could be isolated from the lymph node of an asymptomatic person. The agent was infectious for intraperitoneally inoculated mice with a lethal outcome.[72] More recently, Chung and others[73] isolated *Orientia* from the blood of six individuals who had scrub typhus 1–18 months earlier. The factors that allow for persistent *Orientia* infection are another unresolved topic in scrub typhus research.

Immunity to scrub typhus. Immunity to *O. tsutsugamushi* is complicated by the great antigenic diversity of the agent, the weak and transient cross-protection among divergent strains, and the loss of even homologous immune protection after a few years.[39,76,77] Homologous immunity is provided by antibodies to the major surface protein, a 56 kDa molecule that contains strain-specific epitopes. Antibodies to *O. tsutsugamushi* increase uptake by macrophages and neutrophils and play a role in the clearance of organisms from blood.[72,78–80] Antibodies to the 56 kDa surface protein inhibit entry into target cells and neutralize infectivity.[81] The concentration of *O. tsutsugamushi* strain-specific antibodies wanes over a few years after infection, and heterologous strain protective immune responses last a considerably shorter length of time; therefore, there is the possibility of reinfection.[72,82] Cellular immunity provides cross-protection against divergent strains that is T lymphocyte-dependent. Using an uncharacterized intravenous inoculation mouse model, Jerrells and Osterman[83] showed that depletion of macrophages was detrimental to survival of the mice. Experimental studies of immunity that have used invalid animal models suggest that protective immunity is mediated by Th1 cells. Adoptive transfer of antigen-specific IFN-γ–producing T cells is protective.[84,85] Thus, it is very likely that humoral immunity facilitates phagocytic removal of orientiae when appropriate antibodies are present, and IFN-γ and cytotoxic T lymphocytes play key roles in controlling the infection. Boryong strain-specific immunity stimulated in experimental animals by recombinant Boryong 56 kDa protein correlates with antibodies directed against this antigen.[86]

Patients with scrub typhus have been reported to develop increased serum concentrations of cytokines (IFN-γ, interleukin 1β [IL-1β], IL-12p40, tumor necrosis factor-α [TNF-α], and IL-10), chemokines (CXCL-9 and CXCL-10), and granzymes A and B.[87–92] However, mechanistic studies of protective or deleterious effects of these molecules have not been pursued. Indeed, the roles of CD4 and CD8 T lymphocytes, natural killer (NK) cells, NKT cells, dendritic cells, macrophages, and endothelial cells have not been evaluated in animal models with disseminated endo-

thelial infection that appropriately represents scrub typhus (Table 4).

Attempts to develop a vaccine against *O. tsutsugamushi*. In the past 70 years, numerous approaches have failed to stimulate protective immunity against challenge with *O. tsutsugamushi* to prevent scrub typhus. Approaches have included the use of formalin-killed *Orientia*, inoculation with viable organisms followed by antimicrobial treatment, irradiated *O. tsutsugamushi*, and subunit vaccines.[72,93–96] The results have varied from short-term protection to failure to protect. Protection was frequently defined as preventing death but not protecting from illness. As with natural infection, there was failure to stimulate cross-protection against the many strains circulating in nature. A recent review article has addressed the history of vaccine development for scrub typhus and its current status.[95]

Current approaches should aim to understand why the natural immune response against *Orientia* does not produce sterilizing, long-lasting, and cross-protective immunity. The naturally induced immune response seems inappropriate and is hampered by the immunodominance of antigens encoded by genes that are not conserved across the large number of *Orientia* strains.

Genome sequencing should be pursued, because data from two sequenced strains (Boryong and Ikeda) reveal other potential vaccine antigens (e.g., autotransporter surface Sca proteins) that suggest that a contemporary genomic approach to scrub typhus vaccinology has potential (Table 5).[97]

O. tsutsugamushi and scrub typhus present many unresolved problems that can be addressed by contemporary biomedical scientific approaches. Obligately intracellular bacteria have fallen outside of the scope of bacteriologists and virologists, leading to insufficient effort to undertake the required scientific projects. This situation represents an outstanding opportunity for biomedical scientists to pursue.

TABLE 4
Unresolved issues related to immunity to *O. tsutsugamushi*

Development and validation of appropriate animal models that represent the target cells, pathology, and immune responses of human scrub typhus

Characterization of the early innate immune response to *O. tsutsugamushi* in the mite feeding site, including the effects of chigger salivary proteins and other molecules

Determination of the mechanisms of immunity, including the contributions of T-lymphocyte subsets, dendritic cells, NK cells, cytokines, chemokines, and endothelium

Elucidation of the intracellular orientiacidal mechanisms and potential immunomodulation in infected cells

Explanation for the transience of immune protection

Determination of the prevalence, mechanisms, and consequences of persistent infection

TABLE 5
Unresolved issues related to vaccine development

Identification of the antigens that stimulate long-lasting cross-protective cell-mediated immunity

Determination of the correlates of protective immunity stimulated by natural infection and induced by a protective vaccine

Development of a standardized animal model for detailed vaccine-induced immune response characterization and evaluation

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