International Journal of Entomology Research
www.entomologyjournals.com
ISSN: 2455-4758
Received: 15-06-2025, Accepted: 16-07-2025, Published: 31-07-2025
Volume 10, Issue 7, 2025, Page No. 208-213

Scrub Typhus and *Orientia tsutsugamushi*: Classification, life cycle, and the role of TSA56 in antigenic variation

Govind Sharma, Pragya Sharma, Dr. Devendra Kumar
Department of Zoology, Mohan Lal Sukhadia University, Udaipur, Rajasthan, India

Abstract
Scrub typhus, caused by *Orientia tsutsugamushi*, is a neglected vector-borne disease of major public health concern in the Asia–Pacific region. Unlike other members of the *Rickettsiaceae* family, *O. tsutsugamushi* possesses distinctive biological features, including an obligate intracellular lifestyle, absence of peptidoglycan, and a highly repetitive genome. Transmission occurs via chigger mite larvae, and the bacterium establishes infection by entering host cells, replicating intracellularly, and spreading systemically. Among its surface proteins, the 56-kDa type-specific antigen (TSA56) is the most immunodominant, accounting for up to 15% of total cell proteins. TSA56 plays a crucial role in adhesion through fibronectin binding, immune evasion, and strain-specific antigenic variation. Prototype strains such as Gilliam, Karp, and Kato have served as reference points for studying antigenicity and molecular epidemiology. Understanding TSA56 diversity is essential for improving diagnostic assays, tracking strain circulation, and guiding vaccine development against scrub typhus.

Keywords: *Orientia tsutsugamushi*, Scrub typhus, TSA56 gene, antigenic diversity, prototype strains

Introduction

Scrub typhus is an acute vector-borne zoonotic disease caused by *Orientia tsutsugamushi*, an obligate intracellular bacterium transmitted to humans through the bite of infected larval trombiculid mites (chiggers). The disease is endemic to the Asia-Pacific region, often referred to as the "tsutsugamushi triangle," which accounts for millions of cases annually. Despite its wide distribution and high disease burden, scrub typhus has historically remained a neglected tropical disease, primarily due to its nonspecific clinical features and diagnostic challenges.

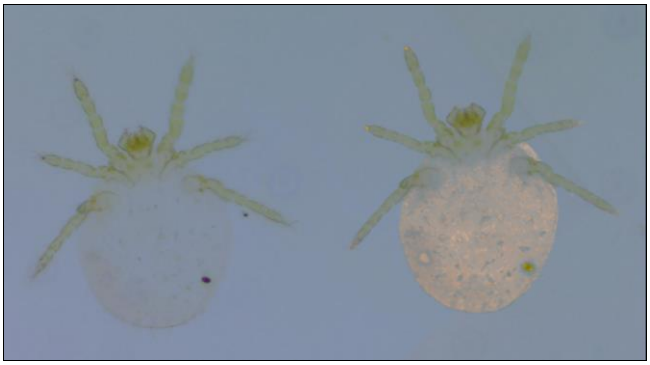

Fig 1: Trombiculid mite (Carrier of *Orientia tsutsugamushi* and vector of Scrub typhus).

The genus *Orientia* is unique within the family *Rickettsiaceae*, showing distinct genetic and structural features that set it apart from related organisms. Its complex life cycle, coupled with antigenic variation among circulating strains, poses significant challenges for diagnosis, treatment, and vaccine development. Among its surface proteins, the 56-kDa type-specific antigen (TSA56) is of particular importance, as it plays a central role in host–pathogen interactions and serves as a key molecular marker for epidemiological and phylogenetic studies.

Epidemiology

Prospective fever cohort studies from Asia identify scrub typhus as a major cause of treatable non-malarial febrile illness. Sero-epidemiological investigations further demonstrate that *Orientia tsutsugamushi* infection is widespread across the region, with reported seroprevalence ranging from 9.3% to 27.9% (median 22.2%; IQR 18.6–25.7). Passive national surveillance systems in South Korea, Japan, China, and Thailand indicate a substantial apparent increase in disease incidence over the past decade, with a median of 4.6 cases per 100,000 population (highest in China: 11.2/100,000/10 years).

Case fatality risks vary by geography and treatment status. In regions with reduced antimicrobial susceptibility, mortality has been reported at 12.2% in South India and 13.6% in northern Thailand. Across studies, the median case fatality rate is approximately 6.0% in untreated cases and 1.4% in treated cases. Severe clinical manifestations are associated with markedly higher mortality: 13.6% in cases with central nervous system involvement, 24.1% in patients with multi-organ dysfunction, and significantly elevated risks of miscarriage and poor neonatal outcomes during pregnancy (Bonell *et al*., 2017).

Classification and General Features

Scrub typhus is caused by the obligatory intracellular bacteria *Orientia tsutsugamushi* (Philip, 1948) [31]. Within the *Rickettsiaceae* family, it is categorised as a distinct genus (Tamura *et al*., 1995) [49]. Based on the homology of the 16S rRNA sequence, phylogenetic analysis was used to show that *O. tsutsugamushi* belongs to the Proteobacteria α-subdivision, indicating that it is a Gram-negative bacterium (Ohashi, 1995) [23]. The bacterium is about 1.2–3.0 µm long and 0.5 µm broad (Tamura *et al*., 1991) [51]. Silverman proposed the existence of a capsule-like layer on the surface of *O. tsutsugamushi* based on electron microscopy studies of the organism treated with an antibody (Silverman &

Wisseman, 1978) [43]. Other studies do not, however, make it very evident.

When inoculated with *Rickettsia akari*, *R. conori*, and *R. typhi*, athymic mice had antibodies reactive with immunizing antigens; but, following infection with *O. tsutsugamushi*, they were unable to produce detectable antibodies (Jerrells & Eisemann, 1983) [12]. This suggests the absence of polymeric antigens with repeated epitopes, such as capsular polysaccharides. The absence of peptidoglycan in *O. tsutsugamushi*'s cell wall explains its total resistance to beta-lactam antibiotics, which are known to block peptidoglycan formation (Amano *et al*., 1987) [1].

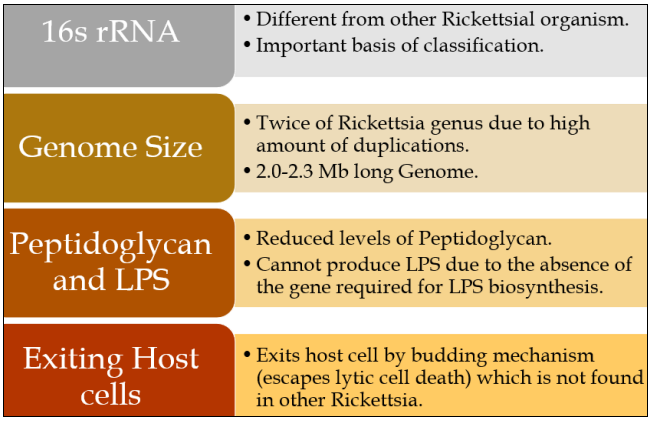

Fig 2: Differences found in Orientia genus from Rickettsia genus.

Orientia genus has three species, *O. tsutsugamushi* which is in context for long, and *O. chuto* which was just identified in a patient with scrub typhus-like symptoms in Dubai (Izzard *et al*., 2010) [11] and *Candidatus Orientia chiloensis* (from Chile) (Balcells *et al*., 2011) [3]. *Orientia* has approx. 2.1 Mb long DNA (Batty *et al*., 2018; Díaz *et al*., 2018) [4, 7]. Its genome is the most highly repetitive bacterial DNA sequenced to date. The reason behind this highly repetitive genome is the high numbers of duplications, deletions, and transposable element rearrangements over the course of evolution.

Life Cycle

Orientia tsutsugamushi has a life cycle that is very different from other *Rickettsiales* order members in many ways. In instance, the nonlytic cellular exit of individual *O. tsutsugamushi* bacteria at the plasma membrane has a striking resemblance to budding of enveloped viruses.

It is found as an endosymbiont in its carrier mites through transovarian and transstadial transfer (Rapmund *et al*., 1969; Sonthayanon *et al*., 2010; Takhampunya *et al*., 2016) [33, 44, 48]. Transmission electron microscopy (TEM) revealed *O. tsutsugamushi* in the cytoplasm of infected mite salivary gland cells (Kadosaka & Kimura, 2003) [14]. It has been demonstrated that the act of mites feeding causes *O. tsutsugamushi* to release from these salivary gland cells, allowing it to enter the acinar lumen and eventually spread to humans and other possible hosts.

O. tsutsugamushi is mostly present in monocytes/macrophages and dendritic cells at the cutaneous site of transmission, where an eschar forms in humans (Paris *et al*., 2012) [30]. As demonstrated in animal models, *O. tsutsugamushi* spreads systemically from the dermal inoculation site (Jiang *et al*., 2018; Keller *et al*., 2014; Soong *et al*., 2016) [13, 15, 45] and can infect endothelial cells, cardiomyocytes, or hepatocytes (Moron *et al*., 2001; Pongponratn *et al*., 1998) [22, 32].

O. tsutsugamushi has developed many strategies to preserve the integrity of the host cell because it depends on undamaged cells for replication. Its capacity to prevent apoptosis in the early phases of infection is one of these. This most likely results from a delay in the mobilization of intracellular Ca2+, which may be caused by bacteria with heat-stable compounds (Kim *et al*., 2002) [18]. It then initiates a proapoptotic gene program that seems to exceed its inherent antiapoptotic activity as the infection worsens (Tantibhedhyangkul *et al*., 2011) [53].

The nonlytic exit at the plasma membrane is another tactic to maintain the integrity of the host cell. Through a process akin to budding, individual bacteria can exit the cell and infect additional cells (Salje, 2021) [35]. It has recently been demonstrated that there are major differences between the intra- and extracellular stages of *O. tsutsugamushi* in terms of shape, transcriptional activity, and protein expression (Atwal *et al*., 2022) [2].

In order to continue replicating in various hosts and host cells, *O. tsutsugamushi* needs to repeatedly escape from membrane compartments during its life cycle: from the phagosome to the cytoplasm, in the cytosol to avoid possible entrapment and autophagosome degradation, and from the cytoplasm into the extracellular space via the plasma membrane.

Entry of *O. tsutsugamushi* inside host cells

Complementary host receptors, extracellular matrix chemicals, and bacterial surface components cooperate to cause bacterial infection of host cells. The most significant extracellular matrix protein thought to be involved in the adhesion and entrance of bacteria into host cells is fibronectin (Fn) (Ozeri *et al*., 1998; Schwarz-Linek *et al*., 2004; Van Putten *et al*., 1998) [28, 38, 55]. With two homologous subunits connected near their C-terminal ends by two disulfide linkages, Fibronectin is an about 250 kDa glycoprotein (Pankov & Yamada, 2002) [29]. The Fibronectin monomer is made up of several functional domains, such as the core cell-binding domain, which has the Arg-Gly-Asp (RGD) motif necessary for interacting with integrins on the cell surface, two heparin-binding regions (called Hep-1 and Hep-2) and a gelatin-binding domain (Ruoslahti, 1988) [34].

O. tsutsugamushi must attach to host cell receptors with high affinity to incorporate itself into the cell, as it is an obligatory intracellular bacterium. *O. tsutsugamushi* shows great binding affinity to Fibronectin immobilised on a solid surface (Lee *et al*., 2008) [20] Research has indicated that the existence of fibronectin enhances the ability of several pathogenic bacteria to invade host cells (Ozeri *et al*., 1998; Schorey *et al*., 1995; Van Putten *et al*., 1998) [28, 37, 55]. Fibronectin incubation significantly boosted the number of infected cells. The infection rate was shown to be reliant on the concentration of fibronectin. (Lee *et al*., 2008) [20].

Fibronectin binds with AD III domain of a bacterial surface TSA56 protein but doesn't show any interaction with AD I and AD II. Specifically, amino acid residues 312 to 341 mediate the interaction with fibronectin, which facilitates internalisation of *O. tsutsugamushi* in the host cell. Using a corresponding peptide reduces bacterial entry in host cell during experiments. This competitive inhibition is the reason behind reduced bacterial concentration inside the host cell (Lee *et al*., 2008) [20].

TSA56 Gene and Antigenic Diversity

Surface-membrane proteins are very important in intracellular bacteria as they assist in host cell entry. *O. tsutsugamushi* has four surface-membrane proteins with molecular weights of 22kDa, 47kDa, 56 kDa, and 110 kDa. 56 kDa MW protein called 56kDa Type Specific Antigen (TSA56) is of the most significance as it is unique to *O. tsutsugamushi* and not produced by any other bacteria (Tamura *et al*., 1985) [50]. It makes up 10–15% of the total cell proteins. This protein is the primary focus of diagnostic procedures such as DNA analysis, ELISA, immunoblotting, and serologic analysis (Stover, Marana, Carter, *et al*., 1990) [46, 47]. This protein assists host cell entry by binding with Fibronectin protein on the host cell surface (Lee *et al*., 2008) [20] and also evades the host's immune reaction. Variation in this protein's antigenic properties and gene sequence contributes to various strains of *O. tsutsugamushi*. This protein has four hyper-variable regions named VD-I to VD-IV resulting in antigenic variation (Ohashi' *et al*., 1996) [24]. Its gene tsa56 has approximately 1550 base pairs and encodes 516–541 amino acid residues (Kelly *et al*., 2009) [16].

TSA56 is an immune-protective antigen and is the target molecule of neutralising antibodies. Nearly every serum antibody produced by scrub typhus patients in the convalescence phase recognizes this antigen. Three antibody-binding domains were identified, called Antigenic Determinant region: Antigenic domain I (AD I); Amino acid: 19 to 113; Antigenic domain II (AD II); Amino acid: 142 to 203; Antigenic domain III (AD III); Amino acid: 243 TO 328 (Seong *et al*., 1997) [39].

Analysis of 56kDa Antigen Gene

As mentioned earlier, other bacteria belonging to the *Rickettsiaceae* family do not express the 56-kDa protein, which is exclusive to one particular kind of bacteria. An analysis of the tsa56 gene locus in other bacteria using molecular data from 11,000 different bacterial genomes revealed that the 56-kDa antigen gene is only found in *O. tsutsugamushi* (Kelly *et al*., 2009) [16]. Numerous investigations that started in the early 1990s published the gene sequences for several *O. tsutsugamushi* protein antigens such as the 56kDa TSA gene of Karp, Gilliam, and Kato strain (Lachumanan *et al*., 1993; Ohashi *et al*., 1990; Stover, Marana, Carter, *et al*., 1990; Stover, Marana, Dasch, *et al*., 1990) [19, 25, 46, 47]. As conventional serotyping was a laborious process that requires antigens and reference serum samples, its applicability is now restricted. Molecular genotyping techniques have shown greater diversity among the strains. A highly desirable target for the development of an *O. tsutsugamushi* vaccine is this protein sequence, which for most of its length shows no apparent similarity to any other protein sequence except the protein's carboxy-terminal end which bears a slight resemblance to a few alphaproteobacteria outer-membrane proteins.

The sequence of the 56-kDa antigen gene seems to be most useful for studies on genetic differentiation amongst *O. tsutsugamushi* strains. Other genes may be useful for studying differentiation within *O. tsutsugamushi* strains, even though no other gene has been found to have levels of variation even somewhat comparable to those observed for the locus encoding the 56-kDa protein. So far, no other locus seems to provide the opportunity for a better analysis of strain variation.

The initial descriptions of this gene locus's sequence suggested that different strains' protein products may differ (Ohashi *et al*., 1990; Stover, Marana, Carter, *et al*., 1990) [25, 46, 47]. To ascertain the evolutionary relationships between strains, a limited set of sequences was compared and the degree of genetic variation was ascertained. Comparing different strains revealed that the protein size varied, with roughly 1550 bases encoding an average of about 520 amino acid residues (Ohashi *et al*., 1989, 1992; Stover, Marana, Carter, *et al*., 1990) [26, 27, 46, 47]. The gene can encode protein sequences with lengths ranging from 541 amino acids to as little as 516. This length variation results from a unique level of nucleotide deletion or insertion within the gene coding frame. These changes occurred mostly in the variable domain regions of the gene. The outcome of this process is the immunological diversity and uncommon substantial amount of protein variation associated with the 56-kDa type-specific antigen.

A comparison of prototype strains revealed multiple regions of hypervariability in the gene (Ohashi *et al*., 1992) [26]. The four regions, known as the variable domains VDI–VDIV (Ohashi *et al*., 1992) [26], roughly match the protein's hydrophilic residue regions (Ohashi *et al*., 1990) [25]. Upon comparing a greater quantity of sequences, these domains typically align with the regions of the protein with the highest degree of sequence divergence between nucleotides and proteins.

Strain variation in *O. tsutsugamushi*

The major outcome of years of continuous research on scrub typhus is ample amount of antigenic strain variations in *O. tsutsugamushi*. The first study on identifying different strains was done by Shishido using the Complement Fixation Test in 1962 (Shishido A., 1962) [42] that was later incorporated by indirect and direct immunofluorescence assay (IFA and DFA). He identified three different types called Prototype isolates; Karp, Kato, and Gilliam. Since that successful attempt, continuous work has been done on identifying new antigenic strains using new and improved technologies e.g. Cross-neutralization, Immunofluorescence Assays, and monoclonal Antibody typing. Later, protein analyses suggested that 56 kDa has high antigenic variability and has a vital role in bacterial entry into the host's body. Since the immunity developed by tsutsugamushi disease is specific to serotypes, an individual recovering from the illness may contract an infection from a different serotype strain. For this reason, it is crucial to understand the predominant serotype in a given location when choosing diagnostic strategies and developing vaccines.

Prototype strains

The term "Prototype strain" refers to early isolates that have been used as a reference for the identification of later found strains. Prototype strains are valuable for experimental studies such as studies of strain virulence, vaccine discovery, pathogenicity, immune system response, etc. As such, three strains found in the 1940s and 1950s named Gilliam, Karp, and Kato are considered original prototype strains of *O. tsutsugamushi*.

Gilliam strain was first identified in the blood of US Public Health Services officer Lt. Col. (Dr.) Alexander Gordon Gilliam (Ecke R. S. *et al*., 1945) [9]. He was posted in the Ledo (Assam) region to assist in the investigation of CBI

fever (China-Burma-India Theater of Operations) (Mackie, 1946) [21]. He managed to survive the disease but for several months, he displayed signs of mental instability and remained "sickly" (Kelly *et al*., 2019) [17].

A soldier named Karp, deployed in the Buna-Gona region was wounded and transferred to a hospital, where he became fragile and was treated with scrub typhus (Derrick E. H. & Brown H. E., 1949) [6] Later this strain named Karp after the soldier, was found very virulent in Guinea pigs and mice, killing mice hosts in 6–10 days. When this blood was inoculated intraperitoneally in the Guinea pig, it killed the Guinea pig 10 days post-inoculation (Kelly *et al*., 2019) [17]. This was the first isolate that was generally available for research purposes and named Karp strain after the soldier (Derrick E. H. & Brown H. E., 1949) [6].

According to Dr. Tsunehisa Suto (80), the Kato strain was isolated in 1952 from a 15-year-old teenager from Kurosawa village, Naka-Kanbara district in Japan. The Kato strain has been retained in JNIH (Japan National Institute of Health) later passed to WRAIR lab of Elisberg (Bozeman & Elisberg, 1967) [5]. Along with Karp, this strain is also considered highly virulent.

Antigenicity of OT strains

With time new Antigenic strains were identified. To date, almost 20 different Antigenic strains have been identified (Kelly *et al*., 2009) [16]. By using the same complement fixation test, Shishido also discovered additional antigenic types distinct from the three prototype strains named 'TA716, TA763, TA678, TH1817, and TA686'. In numerous investigations, these five strains along with three original prototype strains, have served as *O. tsutsugamushi* prototype strains (Dohany *et al*., 1978; Elisberg B. L. *et al*., 1968; Shirai & Wisseman, 1975; Traub & Wisseman Jr., 1974) [8, 10, 41, 54].

A traditional serological approach was used to thoroughly examine the antigenic relationships between these eight prototype strains. The most significant antigens were shared by the strains TA686, TA716, and TA763, and there is an antigenic relationship between the Karp, TA686, TA716, and TA763 strains. Thus, Shirai divided the strains into two groups: the Karp-like group, which included Kato, TA686, TA716, TA763, and TH1817, and the Gilliam-like group, which included Gilliam strain (Shirai A., 1988) [40].

Shimokoshi, Kawasaki, and Kuroki are considered important and are being researched more often. The Gilliam, Karp, and Kato prototype strains differ from these strains antigenically. *Orientia tsutsugamushi* Kuroki strain was obtained from a patient in Kyushu, Japan, and it differs from the prototype strains in size, containing a little larger 58kDa type-specific antigen. This large, type-specific antigenic polypeptide is unique to this strain. Shimokoshi strain is a divergent low virulent strain reported in Japan (Tamura Akira *et al*., 1984) [52] that varies a lot from prototype strains and forms a separate clade on the phylogenetic tree (Kelly *et al*., 2009) [16]

Two new standard serotype Irie/Kawasaki and Hirano/Kuroki are added recently in Kawasaki and Kuroki clade. Hirano strain showed significant cross-reactivity with both Karp and Kato types meanwhile Irie showed the same with Gilliam (Sando *et al*., 2018) [36]

Conclusion

Orientia tsutsugamushi remains a major cause of febrile illness in endemic regions, with its clinical and epidemiological significance linked closely to its unique biology and remarkable antigenic diversity. The 56-kDa type-specific antigen (TSA56) is the most important surface protein, not only facilitating host cell entry but also serving as the primary target for immune recognition, diagnostics, and strain differentiation. The continuous emergence of antigenic variants has hindered the development of long-lasting immunity and an effective universal vaccine, underscoring the importance of ongoing molecular surveillance.

A deeper understanding of the genetic variability of TSA56 and its role in immune evasion is essential for improving diagnostic tools, guiding vaccine design, and monitoring the spread of scrub typhus across diverse geographic regions. Strengthening research on antigenic diversity will remain critical for better disease control and for addressing the growing public health challenge posed by scrub typhus in endemic areas.

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