SUMMARY AND CONCLUSIONS
CHAPTER VI

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6. Summary and Conclusion:

Scrub typhus or tsutsugamushi disease is a febrile illness caused by bacteria of the family Rickettsiaceae and named Orientia tsutsugamushi (formerly known as Rickettsia tsutsugamushi until 1995). It is also called Chigger-borne typhus, tsutsugamushi disease, mite-borne typhus, tropical typhus, rural typhus, Japanese river fever, and Kedani fever. Scrub typhus accounts for up to 23% of all febrile episodes in areas of the Asia-Pacific region where scrub typhus is endemic and can cause up to 35% mortality if left untreated. It is widely distributed throughout Asia, Western Pacific and Australia, stretched out from the Far-east to the Middle-east.

Scrub typhus is transmitted to humans by the bite of the larval stage of trombiculid mites or chiggers, belonging to the genus and subgenus Leptotrombidium. A number of small rodent’s particularly wild rats of subgenus Rattus are the natural hosts for scrub typhus. Orientia is transmitted vertically in mites (particularly Leptotrombidium species) by the transovarial route, and horizontally in rodents through trombiculid larval (chigger) bites. Humans usually become infected when they accidentally encroach ‘mite islands’ that contains infected mites. Mite islands can range in size from a few inches to several meters with a wide range of vegetation types from scrub (tall-growing coarse grass) and primary forest to gardens, beaches, paddy fields, bamboo patches, sandy beaches, rain forests, alpine mountains and oil palm or rubber estates.

O. tsutsugamushi is a member of Gram-negative bacteria, the cells are short rods approximately 0.5 to 0.8 µm in diameter and 1.2 to 3.0 µm long. The genome of Orientia is $1.1-1.5 \times 10^9$ Da and consists of 2,400-2,700 kbp, as determined by pulse field gel electrophoresis. In ultra-thin sections, each cell is surrounded by a cytoplasmic membrane and very soft cell wall showing clear periplasm. In electron microscopy studies the outer leaflet of cell wall is considerably thicker than the inner leaflet and lacks
peptidoglycan and lipopolysaccharide. *Orientia* is very soft and fragile and easily disrupted by light sonication or by osmotic shock. The growth of *Orientia* is more resistant to penicillin. Flagella and endospores are not present. It multiplies by binary fission in the host cell cytoplasm and doubling time is about 9 to 18 h. There is no electron-lucent halo zone around the microbes growing in host cells.

*O. tsutsugamushi* isolates are highly variable in their antigenic properties. The purified *Orientia tsutsugamushi* lysates as determined by SDS-PAGE revealed as many as 30 polypeptides. The major proteins of *Orientia* have the following sizes: 110-kDa, 80-kDa, 70-kDa, 60-kDa, 56-kDa, 47-kDa, 42-kDa, 35-kDa, 28-kDa, and 25 kDa (Tamura et al., 1985). Except for the 70-kDa and 60-kDa proteins, all of these are surface proteins. The 56-kDa protein varies among geographic isolates of *Orientia* and thus called type-specific antigen (TSA). TSA is the most abundant surface protein, probably a structural protein of *O. tsutsugamushi* accounting for about 10 to 15% of its total protein.

Fever typically begins 6-21 days following the bite and is accomplished by maculopapular rash, headache and lymphadenopathy. A typical focal lesion or eschar may develop at the bite site. Acute respiratory distress syndrome (ARDS) is a serious complication of scrub typhus; encephalitis, interstitial pneumonia, myocarditis and pericarditis, acute renal failure, acute hepatic failure, and acute hearing loss can also occur in patients with scrub typhus. In the absence of an eschar, presenting features are often indistinguishable from those of other acute febrile illnesses common in the same geographic region including leptospirosis, murine typhus, and dengue fever.

*O. tsutsugamushi* can be isolated from blood, eschar or tissues. *Orientia* can be grown in the yolk sac of 5-7 day old embryonated chicken eggs, in primary cultured cells of chicken embryos and established cell lines such as HeLa, Vero, BHK, McCoy and L929. Serologically the disease can be diagnosed by using indirect fluorescence assay (IFA), indirect immunoperoxidase assay, enzyme-linked immunosorbent assay (ELISA) and dot blot assay. Molecular diagnosis of scrub typhus can be done by using PCR, real-time PCR and LAMP assays.
In India, the burden of this re-emerging infectious disease is underestimated due to lack of both community-based studies and availability of specific laboratory tests. To our knowledge, incidence of scrub typhus from Andhra Pradesh is unavailable although it has been reported from neighboring states of our country. Disease severity and manifestations vary widely from asymptomatic to fatal and show marked geographical differences. The virulence of *O. tsutsugamushi* differs between strains depending on their serotype. Hence, rapid diagnosis of *O. tsutsugamushi* endemic serotypes are essential to reduce the burden of the disease. The study was aimed at determining the prevalence of sero-positivity for scrub typhus in Southern districts of Andhra Pradesh, comparing the diagnostic utility of Weil-Felix test, ELISA and Nested PCR tests and determining the serotypes of *O. tsutsugamushi* that is clinically relevant here.

As there are no reports from this region regarding the prevalence of Rickettsiosis, so a preliminary study was conducted from January 2011 to February 2012 to know burden of scrub typhus in this area. The Weil-Felix test antigens were prepared in-house. The healthy individuals samples and patients samples were analyzed by Weil-Felix test by using the standard protocol with doubling dilution of 1:20 to 1:320 for initial screening followed by further dilutions (from 1:20 to 1:1280) to achieve end titer. Of the tested 142 samples, 93 (65.49%) were tested positive whereas 49 (34.50%) tested negative for Rickettsiosis. The prevalence of antibodies to scrub typhus was highest followed by typhus fever group and spotted fever group.

A Cross-sectional prospective study was conducted to know the prevalence of scrub typhus and to compare the three diagnostic modalities Weil-Felix test, ELISA and Nested PCR. During the study period from March 2012 to December 2013 a total 113 patients were subjected to Weil-Felix test with *Proteus mirabilis* OXK antigen by using doubling dilution. Among the 113 clinically suspected scrub typhus patient’s, 44 (38.93%) patients were found to be positive by Weil-Felix test. The serum samples were also analyzed by qualitative IgM ELISA. Of the tested samples IgM antibodies to the 56 kDa antigen were detected in 40 (35.39%) patients by IgM ELISA.

The samples that were subjected to serodiagnosis of scrub typhus are also processed by PCR based technique. The DNA extraction was performed with the blood
clot and detection of *O. tsutsugamushi* DNA in blood was performed by Nested polymerase chain reaction targeting the type-specific antigen gene. The PCR conditions were standardized for the amplification of 483 bp type-specific antigen gene product. Of the tested samples 40 (35.39%) samples showed 483 bp DNA fragment corresponding to 56 kDa gene on the agarose gel. The PCR amplified fragment was purified using QIAquick PCR purification kit (Qiagen, USA) and sequenced. The retrieved sequences were submitted to NCBI and the accession numbers assigned to our sequences were KJ740606, KJ740607, and KJ094995. On phylogenetic tree construction the obtained sequences formed a cluster close to Japanese Gilliam (JG) type comprising of Hualien-7, Taitung-2 and UT329.

Along with above 40 N-PCR positive samples 31 additional samples were included for serotyping. The DNA was extracted from the blood clot and Nested PCR was performed to identify the serotypes namely Gilliam, Karp, Kato and Kuroki, Kawasaki with the serotype specific primers and observed on the agarose gel. The PCR conditions were standardized for the amplification specific serotype. The PCR amplified fragment was purified using QIAquick PCR purification kit (Qiagen, USA) and sequenced. The retrieved sequences were submitted to NCBI and the accession numbers assigned to our sequences were KJ094996, KJ094997. The serotypes Karp (230-bp) and Kawasaki (523-bp) was detected among the examined specimens where as other serotypes Gilliam (407-bp), Kuroki (220-bp) and Kato (242-bp) was not identified. It was observed that Karp (71.83%) was the predominant serotype in Southern Andhra Pradesh region followed by Kawasaki (22.53%) serotypes.

In conclusion Weil-Felix test was found to be promising as a screening test for diagnosis of scrub typhus, typhus fever and spotted fever in correlation with clinical feature in a hospital setting where gold standard tests are not available like India. This simple agglutination test can be used all most in all microbiology laboratories across the country to assess the burden of rickettsiosis. ELISA test can be aided as a useful tool in developed laboratories for diagnosing scrub typhus. The nested PCR was found to be a sensitive and reliable method for diagnosing scrub typhus. In suspected patients at acute phase and convalescent phases N-PCR can be used for diagnosis. The
combination of both antibody and molecular assays would be ideal tool for diagnosing scrub typhus. This is the first molecular evidence for scrub typhus in our region. More prospective studies need to be done on this neglected illness, and also bring awareness among the physicians about this re-emerging endemic Rickettsiosis. Serotypes are responsible for variation in clinical presentation by studying circulating serotypes in the endemic regions it would be helpful to develop appropriate vaccine. The results of the present study revealed that two serotypes of *O. tsutsugamushi* are co-circulating in this region. The incidence of combined infection with dual serotypes has also been observed and this may lead to emergence of newer strains with genetic variations that could alter the disease profile in future outbreaks of scrub typhus in this part of India. This information would be useful in understanding the *O. tsutsugamushi* evolution, and may help in correlating disease severity to serotypes or genotypes during future outbreaks. Further studies need to be conducted to analyze the vectors and reservoirs from various parts of India.

It also found that scrub typhus is more prevalent in this region when compared to other rickettsiosis. This study also helps the authorities to undertake therapeutic as well as preventive measures to prevent the morbidity and mortality of scrub typhus. As our region is pilgrim place so many travelers will be coming from other states, so further studies has to be done to understand epidemiological aspects and strain variability of this re-emerging infection. Our results also shows that rickettsial infections are one of the important causes of Pyrexia of unknown origin and active surveillance of rickettsial diseases is required to know exact magnitude and distribution of vector and disease. Further active surveillance of rickettsiosis required to know the exact magnitude of vector and rodents causing rickettsiosis in this region. Thus this study raises awareness about the prevalence of scrub typhus fever and consider it as one of the differential diagnosis of patients with fever from endemic regions of scrub typhus and also emphasizes the need for intervention program to control scrub typhus and mite borne diseases. In conclusion that Weil-Felix test can be used as screening test in primary health centers and ELISA can be used in advanced diagnostic laboratories. However PCR can be aided in confirmation of the positivity.