SERODIAGNOSIS AND MOLECULAR CHARACTERIZATION OF *RICKETTSIA* IN AND AROUND VIJAYAPURA NORTH KARNATAKA, INDIA

A Thesis submitted to the Faculty of Medicine of

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VIJAYAPURA, INDIA.

For the degree of

Doctor of Philosophy

In

Medical Microbiology

By

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CERTIFICATE

This is to certify that the **Mr. Shriharsha Hegde M.L** carried out the work on **"Serodiagnosis and Molecular Characterization of *Rickettsia* in and around Vijayapura, North Karnataka, India''** for the award of **Doctor of Philosophy in Medical Microbiology,** submitted to the **BLDE (Deemed to be University),** Vijayapura, India, for the requisite period, under the regulations in force and this thesis is the bonafide record of the work done by him under my supervision and guidance. This work is original and has not formed before the basis for the award to the candidate of any degree, diploma, associateship, fellowship or any other title.

I further certify that the entire thesis represents the independent work of **Mr. Shriharsha Hegde M.L,** and all the experimental techniques employed in this work are actually undertaken by the candidate himself under my supervision and is worth submitting for the award of the said degree.

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CERTIFICATE

This is to certify that the thesis entitled **"Serodiagnosis and Molecular Characterization of *Rickettsia* in and around Vijayapura, North Karnataka, India''** submitted to **BLDE (Deemed to be University),** Vijayapura, India, for the fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Medical Microbiology** is a bonafide record of the original research work carried out by **Mr. Shriharsha Hegde M.L,** from 2012-2019 under our supervision and guidance in the Department of Microbiology, Shri B.M. Patil Medical College & Research Centre, Vijayapura.

This study represents the independent work conducted by him and has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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DECLARATION

I hereby declare that the thesis entitled **'Serodiagnosis and Molecular Characterization of *Rickettsia* in and around Vijayapura, North Karnataka, India''** submitted to the **BLDE (Deemed to be University),** Vijayapura, India, for the fulfillment of the requirement for the degree of Doctor of Philosophy in Medical Microbiology, is a bonafide record of the original research work done by me under the supervision and guidance of **Dr. Basavaraj V. Peerapur**, Former Professor & HOD, Department of Microbiology, Shri B.M. Patil Medical college & Research Centre, Vijayapura.

This study represents the independent work conducted by me and has not previously formed the basis for award of any degree, diploma, associateship, fellowship or other similar title.

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Name of Guide/Co-investigator Dr.

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Shriharsha Hegde M.L

CONTENTS

ABBRIVIATIONS

ARDS	acute respiratory distress syndrome
LPS	lipopolysaccharide
AD	Antigenic Domain
ALISA	Aptamer based ELISA type assay
ARP	actin-related protein
ARRL	Australian Rickettsial Reference Laboratory
BSL	Biosafety level
CAN	Central nervous system
CD	Clusters of differentiation
CF	complement fixation
DEBONEL	Dermacentor-borne-necrosis-erythema-lymphadenopathy
ELISA	Enzyme linked immunosorbent assay
ESS	erythrocyte-sensitizing substance
G6PD	glucose-6-phosphate dehydrogenase
gltA	Citrate synthase gene
IDT	Intigrated DNA technology
IFA	immunofluorescence assays
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHA	indirect hemagglutination
IL	Interleukin
kDa	Kilodalton
MIF	Microimmunofluorescence assay
MODS	multiorgan dysfunction syndrome

MSF	Mediterranean spotted fever
ompA	Outer membrane protein A
ompB	Outer membrane protein B
PCR	Polymerase chain reaction
RFLP	Restriction Fragment Length Polymorphism
RMSF	Rocky Mountain spotted fever
RNA	Ribose Nucleic Acid
SFGR	Spotted fever group Rickettsiae
SPD,	specificity difference
STG	Scrub typhus group
TG	Typhus group
TIBOLA	Tickborne lymphadenopathy
TSA	Type specific antigen
VD	Variable domain
WF test	Weil felix test

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REAGENTS AND KITS

Sl. No.	Name of the Kit/ Reagent	Manufacturer
1	IgM ELISA for Scrub typhus	Inbios, USA
2	IgM ELISA for R. conorii	Vercell, Spain
3	Master Mix	Promega
4	DNA Extraction Kit	Qiagen
5	Weil felix test kit	Tulip

EQUIPMENT

Sl. No	Name of the Equipment	Make
1	ELISA reader	Tulip
2	Thermal Cycler	ESCO
3	Electrophoresis Unit	Bio Rad, USA
4	Micropipettes	Eppendorf
5	Centrifuge	Remi
6	Deep Freezer	Blue Star

ABSTRACT

Background

Rickettsiosis is the most covert, re-emerging vector borne bacterial disease of public health importance distributed in many parts of the world. The disease is mostly go unnoticed or misdiagnosed due to low manifestation and non availability of specific diagnostic tests at all levels of healthcare setup. Failure of timely diagnosis and treatment leads to significant morbidity and mortality.

Aim & Objectives:

The present study aimed to demonstrate the presence of rickettsial infections in and around Vijayapur district using both serological and molecular diagnostic tests.

Methods:

In the present study, 572 blood samples from clinically suspected patients were screened for the presence of antibodies against rickettsial infections by routinely used Weil felix test. For the serological confirmation, all the serum samples were tested further by more sensitive and specific diagnostic assay, *R.conorii* and Scrub typhus specific IgM ELISA. To detect the etiological agent of the rickettsial infection, blood clot of ELISA positive samples were tested by nested PCR using the primers specific to the gene encoding different antigens of *Rickettsia* and *Orientia*. Sanger sequencing was performed to confirm the amplification of the gene. Phylogenetic tree was constructed to compare the sequences with other strains.

Results:

Out of 572 cases, 213 (37.23%) samples have shown titre of 1:160 and above with Weil felix OX antigens suggestive of seropositivity for rickettsial infection. Among 213 WF positive cases, 180 (31.46) samples have shown agglutination with OX2 antigen suggestive of SFG Rickettsial infection, and 63 (11.01%) cases shown agglutination with OXK antigen suggestive of Scrub typhus infection. In *R. conorii* IgM ELISA, 56 samples

were positive out of 572. In Scrub typhus IgM ELISA, out of 432 samples 23 samples proved to be scrub typhus infection serologically. In nPCR for Scrub typhus, two samples have amplified successfully with the primers specific to 483bp segment of gene encoding 56kDa antigen of *Orientia tsutsugumushi*. Both the samples were sequenced by using Sanger sequencing technique. Sequences of the study sample have shown closest homology with many other strains reported from various parts of India and Southeast Asia. Phylogenetic analysis O. tsutsugumushi with sequences of the study have shown close relatedness with the strain reported from Korea. Several attempts were made to standardize the nested PCR for the detection of *Rickettsia* genus specific citrate synthase gene (*gltA)* and SFGR specific outer membrane protein gene (*ompA)* using specific primers. However, in spite of multiple attempts we could not standardize the PCR.

Conclusion:

Findings of our study demonstrated that Rickettsial infection is also circulating and causing acute febrile illness in and around Vijayapura, North Karnataka region. Significant seropositivity was observed for SFGR than Scrub typhus; however molecular detection of scrub typhus has proved its existence. More seropositivity was observed during the cooler months (August to January). Clinical diagnosis of rickettsial infection is really challenging as most of the common symptoms are non specific, and leads to misdiagnosis if not thoroughly examined. As the routinely used Weil felix test is lacking both sensitivity and specificity, inclusion of more specific tests like IgM ELISA and/or nPCR in routine diagnostic course would be highly beneficial for accurate diagnosis and patient management.

INTRODUCTION

CHAPTER -01

INTRODUCTION

Rickettsiosis is the oldest known vector-borne febrile disease documented since the end of nineteenth century. They are the most covert re-emerging febrile infections of the current era and are prevalent throughout the world due to the cosmopolitan distribution of the vectors that transmit these diseases.[1,33,166.]

Rickettsial infection has been one of the great afflict of mankind, occurring in devastating epidemics during times of war and famine. Napoleon's retreat from Moscow was forced by rickettsial disease breaking out among his troops. Lenin is said to have remarked, in reference to rickettsial disease rampant during Russian revolution, that "either socialism will defeat the louse or the louse will defeat the socialism".[1]

Rickettsial infections are anticipated to be on the increase and have been reported to represent the third most common vector borne disease that is acquired during international travel.[33] They appear to be important causes of fever of unknown origin encountered in travellers returning to their country.[46]

The Rickettsia is named after Howard Taylor Ricketts, the American Pathologist who in 1906 investigated an outbreak and described the transmission of Rocky Mountain spotted fever (RMSF) by the tick, *Dermacentor occidentalis*, later the causative agent for RMSF was identified and named as *Rickettsia rickettsii*.[35] His work was instrumental and laid the foundation for the modern rickettsiology.[36]

Rickettsiosis is caused by a group of small, pleomorphic, nonflagellate, gram negative obligate intracellular parasitic bacterium called Rickettsiae. Initially the

Rickettsia was thought to be phylogenetically occupies a position between bacteria and viruses.[1] Much later, it was confirmed to be a bacteria adapted to obligate intracellular parasitism in eukaryotic cells.[37]

Rickettsial disease is transmitted to humans by the infected ectoparasitic vectors like ticks, mites, lice, fleas and chiggers when the humans accidentally enters in to their habitat and come in contact with their reservoir hosts like rodents. Rickettsiae are associated with arthropods vectors for a small period of their life cycle and are passed to other arthropods by transovarial transmission or horizontal transmission involving vertebrates.[38]

The organism Rickettsia is included in the family Rickettsiaceae of the order Rickettsiales and divided in to two genus namely *Rickettsia* and *Orientia*. Spotted fever group (SFG), Typhus group (TG) belongs to genus *rickettsia* and scrub typhus group belongs to the genus *Orientia*. Advent of newer molecular techniques in the last three decades has facilitated the expansion of genus *rickettsia* and *orientia*, currently 31 species have been identified and more species are being added to the genus every year.[39,40] Spotted fever group rickettsia comprise more than 20 species transmitted by ticks, mites and flea; Typhus group rickettsia includes *Rickettsia typhi* and *Rickettsia prowazekii* transmitted by flea and louse; and Scrub typhus group includes *Orientia tsutsugamushi* and a newly identified species *Orientia chuto* transmitted by chiggers or *Trombiculid* mites.[41]

Introduction of different molecular taxonomic methods in the past decade has made a number changes in the taxonomy of rickettsiae.[42] These taxonomical changes have resulted in the exclusion of numerous other rickettsia-like organisms from being classified as rickettsiae, such as *Coxiella burnetii* and *Bartonella*. These new techniques

also facilitated scientists to study the intracellular bacteria that exhibit few of the phenotypic characteristics traditionally used in taxonomy. Recent proposal of new genetic guidelines have facilitated the reclassification of rickettsial isolates at the genus, group, and species levels by using sequences of the various genes.[43]

Clinical presentation of most of the rickettsial diseases include undifferentiated fever with signs and symptoms similar to the other tropical febrile illness like malaria, leptospirosis, dengue and other viral hemorrhagic fevers.[44,45] Rickettsial diseases usually instigate as an acute, non-specific, febrile illness accompanied by headache, myalgia, nausea and vomiting. In some of the cases a maculopapular rash or even vesicular rash appears on the body after 3-5 days of onset of illness.[46] Presence of an eschar at the site of ectoparasitic arthropod bite, with localized or generalized lymphadenopathy may be the only clinical sign that differentiate mite-borne scrub typhus and tick-borne SFG Rickettsia from other infectious diseases, including murine typhus.[46,47] However, an eschar may not always present and often missed if not thoroughly examined[48,49]. In addition, clinical diagnosis of rickettsiosis is challenging due to the similar signs and symptoms exhibited by other tropical febrile diseases.

Laboratory diagnosis of rickettsial infection is further complicated by non-availability of sensitive and specific diagnostic tests at all levels, mainly in the rural tropics of Asia and other developing countries. Weil Felix test (WF test), the earliest test used to detect rickettsial diseases remains the routinely used test in many countries including India[44,50] Over the past few decades, newer diagnostic assays for the detection of rickettsial diseases are available, mainly in the developed countries. These newer assays were not available in developing or under developed countries until recently as they are expensive, need well equipped laboratory setup and non availability of kits and reagents locally.[51]

Rickettsial infections are escalating and are widespread right through the world except Antartica. SFG rickettsiae and TG rickettsiae cases have been reported from several geographical parts of the world. However tstsugumushi disease or Scrub typhus is geographically widely endemic in a confined area of the Asia- Pacific region, distributed in tsutsugamushi-triangle i.e. India and Nepal in the west; China, Japan, South Korea, and Taiwan in the north, and Australia and Indonesia in the south [2,3,4]. Recent studies have reported the existence of new species of scrub typhus in other parts of the world also. In Indian subcontinent the presence of rickettsial disease particularly that of scrub typhus and Indian tick typhus have been reported from Jammu & Kashmir,[159] Himachal Pradesh,[21] Uttaranchal,[1] Uttara Pradesh,[6] Rajasthan,[26] Delhi,[10] Assam,[7] West Bengal,[161] Maharastra,[1] Andra Pradesh,[4,12] Karnataka,[3,13,14] Tamil Nadu,[2,8,23] Pondicherry,[2,7,16,22] and Kerala.[17]

Past two decades, several researchers from different geographical parts of India have reported seropositivity of Rickettsial infections using single or combination of serology based tests like ELISA, Weil felix and Microimmunofluorescence assay etc. [7,12-14, 19-21,31] Further, a few researchers have also demonstrated the PCR, a molecular test for the diagnosis of Rickettsial infection by detecting different genes from blood samples and vectors.[16, 27-30,32]

Though more information is available today, the whole picture is still not clear regarding *Rickettsia*, and more knowledge is needed if we are to be able to monitor emerging diseases, perform accurate and prompt diagnoses and develop better tools to study the bacteria and their pathological effects on humans and animals.

AIM AND OBJECTIVES

CHAPTER - 02
AIM AND OBJECTIVES

2.1. Aim

The study aims to investigate the presence of Rickettsial infections in & around Vijayapura using serological and molecular methods, identification and characterization of the circulating rickettsial species for the effective patient management.

2.2. Objectives

1. Screening of samples collected from Patients with Pyrexia of unknown origin for the evidence of Rickettsial infection serologically by Weil – Felix test.
2. Serological confirmation of rickettsial infections by IgM ELISA.
3. Identification and Characterization of the circulating Rickettsial species by molecular methods.

REVIEW OF LITERATURE

CHAPTER-03

REVIEW OF LITERATURE

3.1. History

Rickettsiosis is the oldest known arthropod-borne diseases, documented since the end of the 19th century.[52] Howard T Ricketts, an American Pathologist who in 1906 described the first rickettsial organism associated with the disease rocky mountain spotted fever (RMSF) transmitted by the tick, *Dermacentor occidentalis*.[35,53] His efforts was instrumental in paying the way for the modern rickettsiology.[36] Most significant findings were the roles that ectoparasites found to play as vectors of rickettsial infection and reservoirs of rickettsial organisms in an endemic area. In 1916, Rocha Lima with his friend Czech protozoologist Stanislaus Matthias Von Prowazek has proposed the genus name rickettsia. They studied the etiology of typhus fever, and proved that *R. prowazekii* is the causative agent of epidemic typhus.

Kitashima and Miyajina from Japan in 1918 have studied the etiological agent of scrub typhus or "tsutsuga" disease found along the banks of the upper tributaries of the Shinano River, transmitted by small mites. They found significant similarities in the symptomatology and mechanism of scrub typhus with RMSF, and later concluded that scrub typhus belonged to the same group as the agents of RMSF and typhus.[54] The first identification of the causative agent of scrub typhus was by Nagayo and co-workers in 1930 and named the organism as *Rickettsia orientalis*. [55] Later Ogata in 1931 proposed the name *Rickettsia tsutsugamushi*.

Initially *Rickettsia* was classified as a virus or an organism somewhere between viruses and bacteria.[52] Much later, it was established to be an intracellular bacteria. [37,56]

Comprehensive researches by the several researchers have demonstrated that the rickettsial life cycle involves both vertebrate and invertebrate hosts. Not only do haematophagous arthropods play an important role as vectors, they are also a primary reservoir and amplifying host, together with some small mammals such as rats and opossums.[38]

3.2. Classification:

Before the use of 16S rRNA sequence to determine the phylogenetic relationship between prokaryotes in the order *Rickettsiales*, a scrupulous set of criteria for classical taxonomy was used. In the evolutionary tree described by Bergey's Manual of Systematic Bacteriology in 1939, the rickettsiaceae family consisted of four genera, namely *Rickettsia*, *Cowdria, Ehrlichia* and *Coxiella*, and the position of *Rickettsia spp*. was widely accepted. The order *Rickettsiales* published in the eighth edition of Bergey's manual of systematic bacteriology in 1974 consisted of three families; *Rickettsiaceae, Anaplasmataceae* and *Bartonellaceae*.[61] As a result of the introduction of molecular taxonomic methods such as 16S rRNA analysis, classical taxonomy used was revised and a number of major changes to the taxonomy of *Rickettsia* have been made in the past decade.[42] These new techniques enabled scientists to study intracellular bacteria that express few of the phenotypic characteristics traditionally used in taxonomy and which helped in the exclusion of a many of other organisms from being classified as rickettsiae (Figure 01).

1n 1989, based on the 16s gene sequence analysis, *Coxiella* was removed from the family *Rickettsiaceae* followed by removal of the *Bartonella spp.* in 1993.[62] After the reclassification, the *Rickettsia* was placed in the α-1 subgroup of *Proteobacteria*, while

Bartonella is in the α-2 subgroup. The *Coxiella burnetii* (originally *Rickettsia burnetii*) placed in γ subgroup due to the findings that its 16S RNA sequence is more similar to members of the γ subgroup and it is no longer viewed as a true *Rickettsia*.[63]

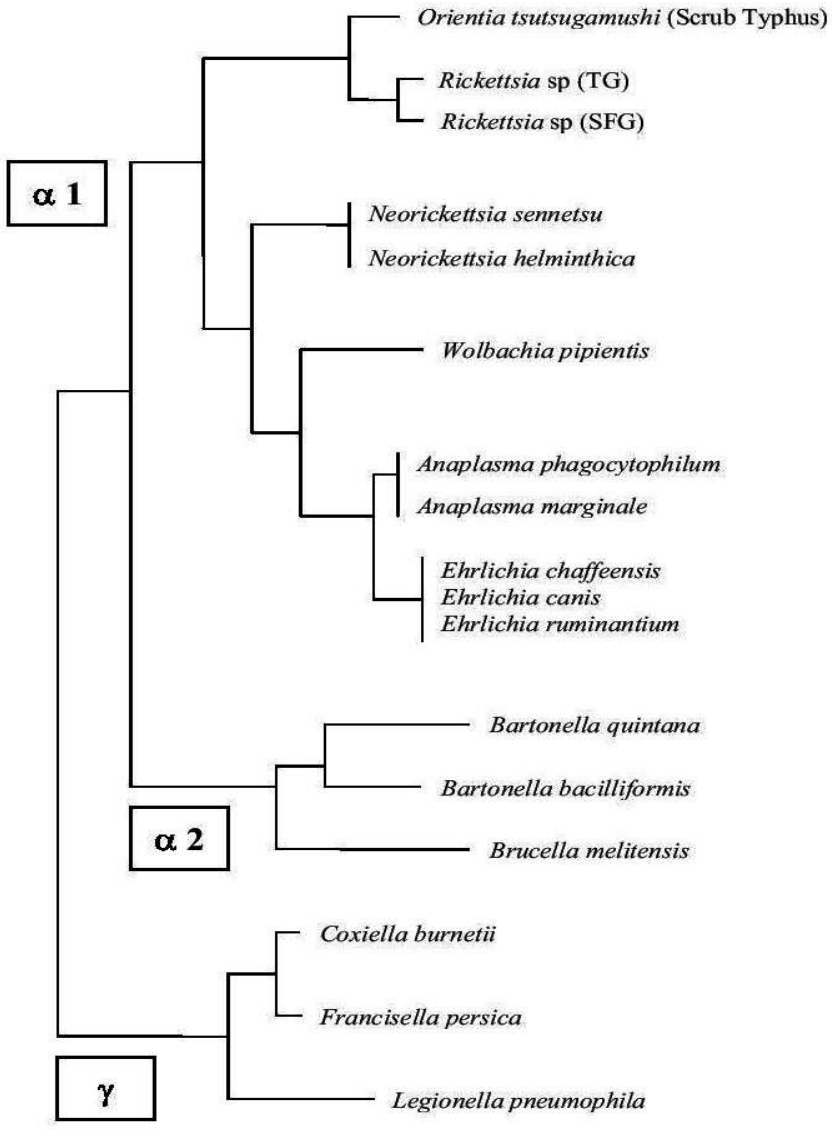


Figure 01: Tree illustrating genetic relationships between the different rickettsial/rickettsia-like organisms found in three different *Proteobacteria* divisions adapted from JS Dumler *et al.* 2001.[64]

Traditionally, the rickettsial isolates are classified into three groups, spotted fever group rickettsiae (SFG), typhus group rickettsiae (TG) and scrub typhus (STG) based on their physiological characteristics of intracellular localization, optimal growth temperature, antigenic properties i.e. cross reactivity of patient sera with somatic (O) antigens of *Proteus* strains (Weil-Felix test) and vector involved in transmission.[65] In 1995, the scrub typhus group was removed from genus *Rickettsia* and reclassified into the genus *Orientia*, and is placed in the same α-1 subgroup as the genus *Rickettsia*.[66] Recently, another two groups have been proposed under the genus *Rickettsia* namely transitional group, constituting *R. akari*, *R. australis*, and *R. felis* [67,68] and, Ancestral Group (AG) consisting of *R. bellii* and *R. canadensis*.[69-71]

Recently Fournier *et al.* have proposed a new genetic guidelines for the reclassification of rickettsial isolates at the genus, group, and species levels by using sequences of the various well described genes i.e. *rrs* (encodes 16S rDNA), and four protein-coding genes; *gltA* (encodes citrate synthase), *ompA* (encodes *rOmpA*), *ompB* (encodes *rOmpB*) and *sca4* (encodes *gene D*).[43,73]

With the increased addition of newer species in the genus *Rickettsia* and *Orientia*, the International committee of expert rickettsiologists recently proposed a polyphasic approach to categorize the rickettsial and related bacteria based on phenotypic, genotypic and phylogenetic criteria.[72]

In the polyphasic classification of the rickettsial isolates, the first step is to study the rrs and *gltA* gene homologies of the isolate. If a homology of >98.1% for *rrs* gene and >86.5% for *gltA* gene is observed with at least one validated *Rickettsia species*, it could potentially be a rickettsial species, otherwise a genus other than *Rickettsia*. In the second

step, the gene homologies for, the *rrs*, *gltA*, *ompA*, *ompB*, and *sca4* are studied. If more than one criteria is fulfilled, the isolate may belong to the validated rickettsial species, if not, potentially new species. In the third step, the *Rickettsia* is tried to isolate in pure culture. For potential new species, if no growth is seen it could be considered as *candidatus*, if growth is positive it could be a new species. When the isolate belong to validated species, if pure growth could be obtained, strain typing is done using serotyping (SPD) and epidemio clinical characteristics. If positive for mouse serotyping (≥3), it is considered as sub species. If the isolate belonged to the validated species in step 2 and could not be grown or ≥3SPD was not observed, then the strain of the species is identified as a known species based on step 1 and 2. In case of the new species and subspecies, the phenotypic characteristics must be described, and the type strain is deposited into two independent official culture collections and must be published in *Int. J. Syst. Evol. Microbiol.* or in another peer reviewed journal and in a validation list in *Int. J. Syst. Evol. Microbiol*. As a result of introduction of new identification and classification methods, today the family *Rickettsiaceae* consists of more than 31 defined species and subspecies (Figure 02).

3.3. Genetic characteristics

Genome sizes of rickettsial isolates are relatively much smaller than those of other bacteria. The circular genome ranges in size from 1.1 to 1.6 million base pairs. Rickettsia DNA has a low G+C content (29-33%), coding regions range from 75.2% (R. canadensis) to 85.2% (R. belii). Genomic analysis of the eukaryotic mitochondrial genome suggests a close relationship between rickettsiae and mitochondria.[71, 74-76] Based on phylogenetic

analysis, mitochondria have been shown to have evolved from the α-proteobacteria and more specifically from Rickettsiaceae.[75,77]

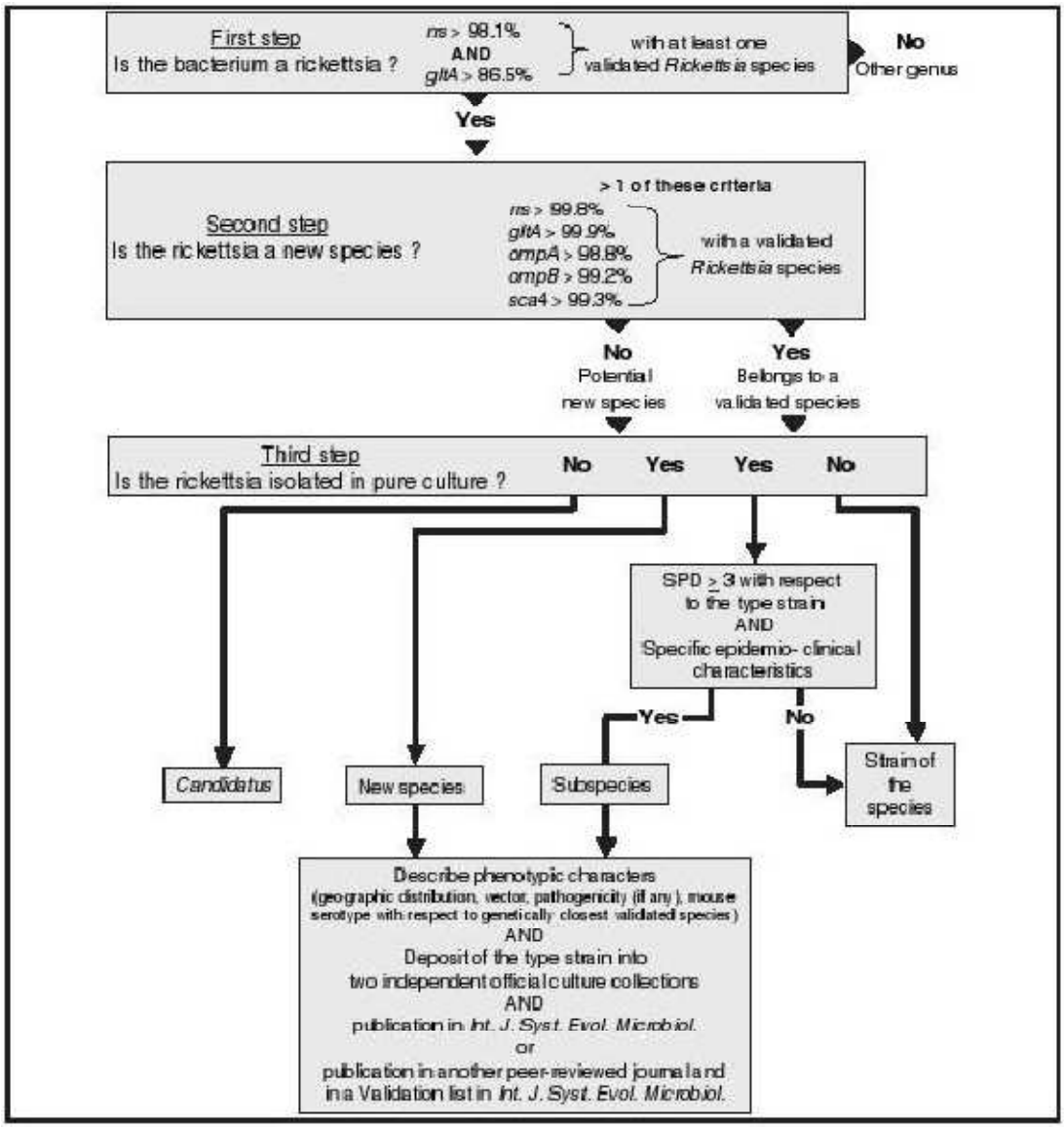


Fig-02: Polyphasic classification of the Rickettsial isolates adapted from Fournier et al.[73]

The genome seems to be in constant reduction, as the intracellular lifestyle enables use of the host cell machinery and it is possible that redundant genes are degraded. This is thought to reduce the bacteria's fitness in relation to their arthropod host and to cause them to become more virulent for both arthropods and humans. The

underlying mechanism is still unknown, but has been suggested to involve loss of regulatory genes.[75,78]

The first rickettsial plasmid was discovered in *R. felis* in the year 2005.[79] The two plasmids identified in the *R. felis* are *pRF* and *pRFδ* with base pair sizes 62,829 bp and of 39,263 bp respectively. The size of the main circular chromosome is 1,485,148 bp.[80] Since the first description, the number of *Rickettsia spp*. reported to be having plasmids has increased rapidly. Today many of the *Rickettsia spp*. have been whole genome sequenced, which has led to a vast amount of data not only on protein coding genes, but also on all genetic information including the discovery of plasmids. Some of the *Rickettsia spp*. which has been whole genome sequenced is *R. helvetica*, *R. monacensis*, *R. massiliae*, *R. akari*, *R. africae*, *R. Canadensis*, *R. prowazekii*, *R. typhi*, R. felis, *R. rickettsi*, *R. conorii* and *R. sibirica*. [76,81-84] However, high passaged strains might lose their plasmids, which could explain the long-held belief that *Rickettsia spp*. lack plasmids. The plasmids are thought to code for a type IV secretion system, a heat chock protein and an antigenic cell surface protein. Plasmids could be an explanation for the genetic diversity and adaptability of the genus, despite rickettsia's reduced genome.[79,81-84] With the discovery of rickettsial plasmids, it is now better understood how conjugative genes, widespread in the genus, are horizontally transferred.[85] The presence of widespread and potentially mobile plasmids in *Rickettsia* has epidemiologic and evolutionary implications.

3.4. Rickettsiosis- Etiology, transmission and distribution

Rickettsiosis is a zoonotic vector borne febrile infection with rashes, headache, myalgia, nausea and vomiting. The disease is caused by the bacterium belonging to the family *Rickettsiaceae* and distributed worldwide.[58] The disease is transmitted by the arthropod vectors like tick, mite, lice and flea. The causative organism is gram-negative pleomorphic coccobacilli belonging to the division *α- proteobacteria*. *Rickettsia* species are strictly intracellular bacteria. This affects the analysis methods available, because cultivation must take place in living cells and cannot be carried out on agar plates or in broth. The bacteria are small, 1.5 - 2 μm in length and 0.3 - 0.7μm in diameter, and have a typical Gram-negative cell wall on the outside of a thin cytoplasmic membrane. Outside the cell wall is a slime layer or capsule built up by lipopolysaccharide, characterized as a halo around the bacterium when imaged by electron microscopy. *Rickettsia spp*. multiply by transverse binary fission, and when the bacteria are starved the division is halted and the bacteria become abnormally long rods.[56,59,60]

Table-01: Rickettsial organisms known to cause human diseases, mode of transmission and their distribution adapted from William L. Nicholson *et al.*, Chapter 7(78) Travelers' Health, CDC (https://wwwnc.cdc.gov/travel/yellowbook/2020).[304]

Rickettsial Organism	Disease caused	Name of the vector	Animal reservoir	Geographical distribution	Ref
Spotted Fever Group (SFG) Rickettsiae					
Rickettsia rickettsii	Rocky Mountain spotted fever	Ticks *Dermacentor andersoni, Dermacentor variabilis*	Rodents	Western Hemisphere	35,86
Rickettsia conorii Subsp. *conorii*	Mediterranean spotted fever	Tick *Rhipicephalus sanguineus*	Dogs, rodents	South Europe, Africa, Middle East	87,88
Rickettsia conorii Subsp. *indica*	Indian tick typhus	Tick *Rhipicephalus sanguineus*		India	89
Rickettsia conorii Subsp. *israelensis*	Israeli Spotted fever	Tick *Rhipicephalus sanguineus*		Israel, Western Asia, Tunisia	90
Rickettsia conorii Subsp. *caspia*	Astrakhan fever	Ticks Rhipicephalus sanguineus Rhipicephalus pumilio		Astrakhan, a region of Russia	91,92

Rickettsia africae	African tick bite fever	Ticks *Amblyomma hebraeum* *Amblyomma variegatum*	Ruminants	Sub-Saharan Africa, Caribbean	93,94
Rickettsia parkeri	Maculatum infection American tick bite fever	Ticks *Amblyomma maculatum* *Amblyomma americanum* *Amblyomma triste*	Rodents	North and South America	95
Rickettsia siberica	Siberian tick typhus, North Asian tick typhus	Ticks *Dermacentor nuttali* *Dermacentor marginatus* *Dermacentor silvarum* *Haemaphysalis concinna*	Rodents	Asia, Europe, Africa	96,97
Rickettsia japonica	Japanese spotted fever	Ticks *Ixodes ovatus* *Dermacentor taiwanensis* *Haemaphysalis longicornis*	Rodents	Japan	98,99,100
Rickettsia honei strain *"marmionii"*	Flinders Island spotted fever, Thai tick typhus	Ticks *Bothriocroton hydrosauri,* *Amblyomma cajennense,* *Ixodes granulatus*	Rodents, reptiles	Australia, Thailand	101,102

Rickettsia slovaca	Tick-borne lymphadenopathy TIBOLA, DEBONEL	Ticks *Dermacentor marginatus* *Dermacentor reticulatus*	European boar, Lagomorphs and rodents	Eastern and Southern Europe, Asia and recently found in US tick colony	103,104
Rickettsia massiliae	Mediterranean spotted fever-like disease	Ticks *Rhipicephalus sanguineus* *Rhipicephalus turanicus* *Rhipicephalus muhsamae*	unidentified, possibly the dogs	Spain, France, Greece, Switzerland, Portugal, Sicily, Mali, United States and central Africa	105,106, 107
Rickettsia heilongjiangensis	Far Eastern spotted fever	Tick *Dermacentor silvarum*	Rodents	Northern China, Far East of Russia and eastern Asia	43,108
Rickettsia mongolotimonae	Lymphangitis Associated Rickettsiosis	*Tick* *H. truncatum*	Rodents	Mangolia, southern France, South Africa's Northern Province Portugal, China,	109
Rickettsia Helvetica	Aneruptive fever	Tick *Ixodes persulcatus*	Rodents	Central and northern Europe, Asia	110
Rickettsia monacensis	Mediterranean spotted fever-like disease	Tick *Ixodes ricinus*	Lizards, possibly birds	North Africa and Europe	

Rickettsia raoultii	TIBOLA and DEBONEL	Tick *Dermacentor andersoni Rhipicephalus sanguineus Amblyomma americanum*	Unknown	Europe, Asia 1999	111
Rickettsia aeschlimannii	Rickettsiosis	Tick *Amblyomma variegatum, Hyalomma marginatum*	Unknown	South Africa, Morocco, Mediterranean littoral	112
Typhus Group (TG) Rickettsiae					
Rickettsia prowazekii	Epidemic typhus	Human body louse, ectoparasites of flying squirrel	Human, flying squirrels	Asia; North, Central and South America and Central Africa	113
Rickettsia typhi	Murine typhus	Flea	Rodents	Temperate, tropical and subtropical areas worldwide	113
Ancestral Group (AG) Rickettsiae					
Rickettsia bellii	*Unknown*	*Ixodids* (hard ticks) *and Argasids* (soft ticks)	Wild rodents	United States and South America	114
Rickettsia canadensis	RMSF-like disease	Ticks *Haemaphysalis rispalustris*	Rabbits	Canada, USA McKiel *et al in 1962-63*	McKiel *et al in 1962-63*

Transitional Group (TRG) Rickettsiae					
Rickettsia felis	Cat flea borne rickettsiosis	Flea	Rodents, Opossums and	North and South America, Europe, Asia and Africa	115,116
Rickettsia akari	Rickettsial pox	Mite	Wild rodents and House mice	Turkey, , South Africa, Korea, Countries of the former Soviet Union, North and South America, Balkan countries,	117,118
Rickettsia australis	Queensland tick typhus	Ticks *Ixodes holocyclus* *Ixodes tasmani*	Rodents	Australia Tasmania	119,120
Scrub Typhus Group					
Orientia tsutsugamushi	Scrub typhus	Chigger (Larval stage of mite)	Rodents	Northeast Asia, Northern part of Australia, recently recognized in Chile as well as in some African countries	121, 66
Orientia chuto	Scrub typhus	Unknown	Unknown	United Arab Emirates	41

3.4.1. Genus *Rickettsia*

Currently, the genus *Rickettsia* is divided into 4 groups; spotted fever group (SFGR) the typhus group (TGR), two newly formed the ancestral group and the transitional group. Geographically, Rickettsial diseases have been reported worldwide, however, each *Rickettsia spp.* is associated with a defined geographic area, although more than one species may be found circulating in a particular region.[308]

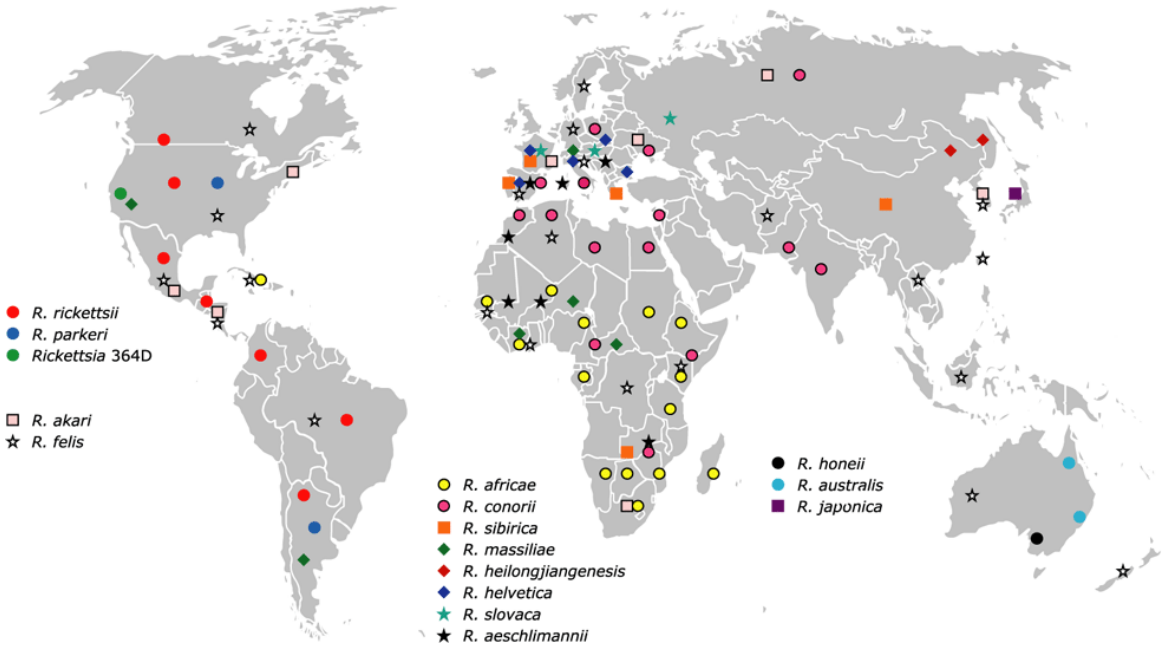


Fig -03: Geographical distribution of known pathogenic rickettsiae belongs to genus rickettsia.

3.4.1.1. Spotted Fever Group (SFG) Rickettsiae

The spotted fever group rickettsiae are of major clinical significance to humans. The members of SFGR live and multiply in both nucleus and free form in the cytoplasm of their mammalian and arthropod host cells. Members of SFG Rickettsiae have a trilaminar cell wall with a periplasmic space separating the cell wall from the

cytoplasm.[56,59] Species-specific soluble antigens are located on a micro capsular layer on the outer leaflet of the cell wall. There are currently more than 20 recognized species/strains, and of which at least 13 are recognized as cause of rickettsioses in humans.[124] With the recent proposals for two new groups under the genus *Rickettsia*, presently the SFG rickettsiae is exclusively tick borne.[68] The rickettsial disease occurs on all continents except Antarctica, but follows the spread of their respective vectors. The distribution of SFGR species is more geographically restricted due to the specific habitats of their tick vectors and reservoirs. Most of the SFGR species are recognized by the name of the geographical area from where the first clinical description of disease reported.[70,125] Clinical manifestation may vary between species and geographic regions. Many epidemiological and serodiagnostic studies from various parts of Southeast Asia have reported that, *R. conorii* subsp. *indica* (causative agent of Indian tick typhus), *R. helvetica*, *R. honei*, *R. japonica*, and flea-borne *R. felis* are the major rickettsial organisms causing human diseases in Southeast Asia region.[57] Throughout Southeast Asia, infections are mostly occur in rural areas where human and animal interactions are more common, however, some cases have been reported from urban areas also. One clinical case has been reported from urban Bangkok, Thailand, without any antecedent exposure to rural setting.[126]

In India, although spotted fever group rickettsiae (Indian Tick Typhus) was clinically described at the beginning of the twentieth century, the specific strain of SFGR which was assumed to cause Indian tick typhus has never been isolated from the patients, nor proved by strain specific serological tests. In 1950, a SFG *Rickettsia* isolate was recovered from a brown dog tick, *Rhipicephalus sanguineus,* collected in India and

assumed to be the etiological agent of the disease Indian tick typhus. It was studied further and identified as *Rickettsia conorii*, the etiological agent of Mediterranean spotted fever, which are reported from all around the Mediterranean and found to be transmitted by the same vector species. However, picture of the disease reported in India differs from the common description of Mediterranean spotted fever. The appearance of rash on the patient's body is often purpuric, and presence of an eschar at the inoculation site or bite site is rarely found in majority of cases. The disease severity in India is reported to be mild to moderately severe.[89,305]

Serological evidence of SFG rickettsial infections have been reported from various states of India like Jammu & Kashmir,[2] Himachal Pradesh,[3] Uttara Pradesh,[4,5] Haryana,[6] Rajasthan,[2] Assam,[7] West Bengal,[2] Maharastra,[8,9]Andra Pradesh,[16] Karnataka,[10-13] Tamil Nadu,[14,15]Kerala,[17] Puducherry[18] and Manipur[2] in the past 10-15 years.

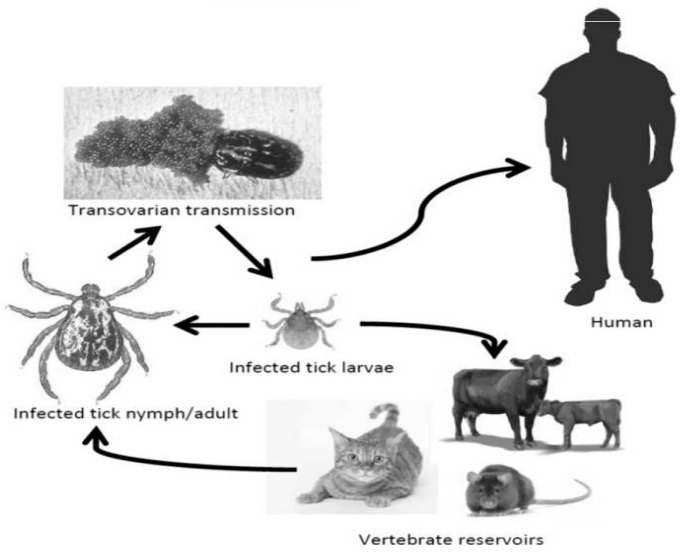


Fig-04: Life cycle and mode of transmission of SFG rickettsiae adapted from Aung *et al*., *Am. J. Trop. Med. Hyg 2014* [57]

3.4.1.2. Typhus Group (TG) Rickettsiae

Rickettsia prowazekii and *Rickettsia typhi* are the only two rickettsial organisms classified within TG rickettsiae so far. The disease caused by the *Rickettsia prowazekii* is primary louse-borne typhus transmitted by the human body louse *Pediculus humanus*. Brill-Zinsser disease (relapsing louse-borne typhus) is revival of primary louse-borne epidemic typhus fever, except that patients who do not develop immunity during primary infection. *Rickettsia typhi* is responsible for the disease Murine typhus transmitted by the vector rat flea *Xenopsylla cheopis* [127,128] and cat flea *Ctenocephalides felis*.[129-132] Fleas normally amplify in markets, grain stores, breweries, and garbage depots where rats (mainly *R. rattus* or *R. norvegicus*) serve as the main reservoir. However, other rodent species and cats have also been implicated as reservoirs for fleas. The disease is transmitted to humans through infected louse feces rubbed into broken skin or mucous membranes or inhaled as aerosol, and *R. typhi* found to be remain infectious in dried flea feces for >100 days.[46,129,133] The members of TG Rickettsiae exist in free form in the cytoplasm of their mammalian and arthropod host cells. Unlike SFG rickettsiae, TG rickettsiae cannot pass through the nuclear membrane of their host cell.

Murine typhus usually occurs in tropical and temperate climates[133], however, occurrences of this disease reported from worldwide due to the wide distribution of vectors louse and flea. In south east Asia, infections have been reported among residents of cities in more wealthy countries such as Singapore, and immigrant workers living in deprived and unhygienic conditions seemed to be at a higher risk.[129,133-135] However, unlike other rickettsial diseases, no seasonal variation is observed with murine typhus. [134,

[136-137] In Indian population, typhus group rickettsiosis have been reported from Uttar Pradesh[4], Kashmir[19], North East region[20] in last decade.

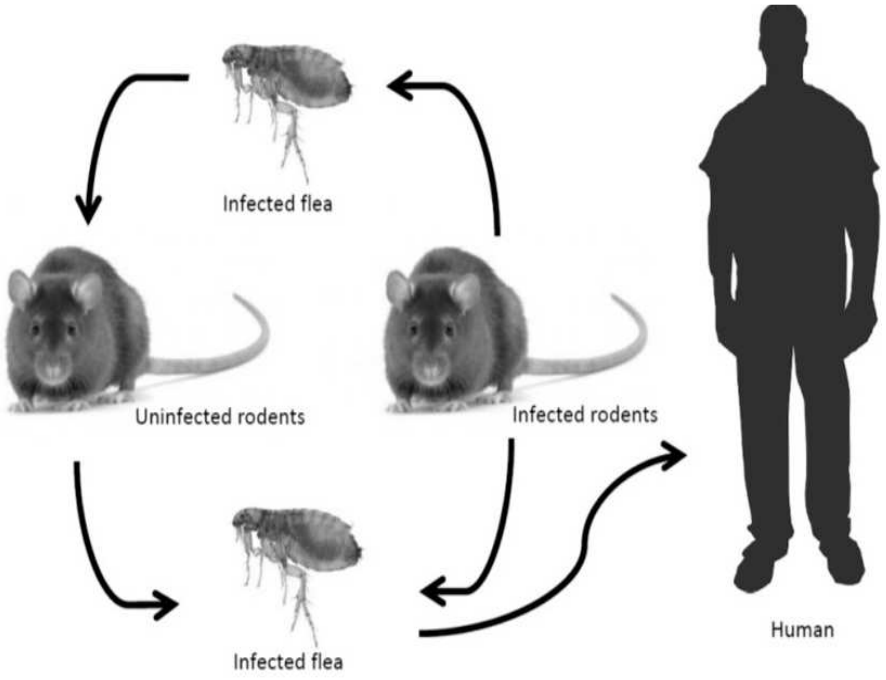


Fig -05: A simplified diagram showing the life cycle of Typhus group rickettsiae adapted from *Aung et al., Am. J. Trop. Med. Hyg 2014* [57]

3.4.1.3. Ancestral Group (AG) Rickettsiae

Ancestral group rickettsiae (AG) consist of two rickettsial organisms i.e. *Rickettsia bellii* and *Rickettsia Canadensis*. Formerly both the species were included in the SFGR as they are tick borne and demonstrated to share common characteristics with both SFG and TG rickettsiae described by Stothard *et al.* in1994.[138] Comparison of the 23S *rRNA* sequences with *R. rickettsii* and *R. prowazekii* indicates that R. bellii and *Rickettsia Canadensis* exhibit unique characteristics of its own and predates the divergence that created the SFG and TG genera.[139]

3.4.1.4. Transitional Group (TRG) Rickettsiae

The transitional group was proposed by Gillespie *et al*. in 2007,[68] which consists of *Rickettsia akari*, *Rickettsia australis* and *Rickettsia felis*. The mite-borne *Rickettsia akari* causes Rickettsial pox in humans, and distributed in Countries like South Africa, Turkey, former Soviet Union, Korea, Balkan countries, North and South America. Animal reservoirs are house mice, wild rodents. The tick- borne *Rickettsia australis* is the causative agent for the North Queensland tick typhus reported from Australia. The reservoirs are rodents and geographically distributed in Australia, Tasmania. The flea-borne *Rickettsia felis* is responsible for the flea-borne spotted fever in humans and cases are reported from North and South America, Europe, Africa and Asia. The main animal reservoirs domestic cats, rodents, opossums. [140] Both *Rickettsia akari* and *Rickettsia felis* are described as being phenotypically and genotypically divergent enough from SFG and TG rickettsiae, to create their own grouping. Genotypic analysis supports this hypothesis, showing that genes found in members of TRG were possibly acquired through conjugational exchanges with AG rickettsiae. [68] However, few authors have reported *R. felis* as still a member of SFG rickettsiae on the basis of the presence of *ompA* gene.[129,141] *Rickettsia australis* was recently described phylogenetically as a member of TRG,[142] however it's LPS is antigenically similar to SFGR.

3.4.1.5. Scrub typhus

The disease scrub typhus has historical significance, as the evidence of this disease is found in China as early as 313 A.D. Later, descriptions were found in Japan in the early 1800's; however, until 1926 the disease was undifferentiated from other rickettsial diseases of that time.[143] The different names of the causative agent were

Rickettsia orientalis,[144] *Rickettsia tsutsugamushi*[145] and the current accepted name is *O. tsutsugamushi*.[66] The disease came to the limelight only during the II world war.[146] The British forces felt the impact of scrub typhus on military during their stay in Burma experiencing 110 cases of scrub typhus in 1934. At one point of time, scrub typhus ranked only behind malaria as an important human infectious disease. In India, the earliest reported cases of scrub typhus were among the US army men at the 100th Station Hospital in New Delhi. Further, many cases were also reported in the US troops at Assam, producing higher mortality than any other area during the warfare. The other causes of increased mortality were concomitant diseases such as dysentery, malaria and prolonged stress due to continuous combat.[147]

Orientia tsutsugamushi causes a complex and potentially life-threatening disease to humans known as scrub typhus or 'tsutsugumushi' disease. The infection is transmitted through the 'chiggers' the larval stage of mites belongs to the family *Trombiculidae*.

Natural hosts for scrub typhus are the small rodents particularly wild rats. The vector is found in different ecological niches such as semi deserts, equatorial rainforests and Alpine subarctic territory in the Himalayan regions. Scrub typhus can occur in areas which are inhabited by chiggers. Endemic foci are usually connected with specific habitats such as abandoned plantations, gardens or rice fields, dense forest clearings, shrubby periphery of fields and forests, river banks and grassy fields.[148-150] These ecological territories which attract the natural host of mite vectors are called 'mite islands'.[151]

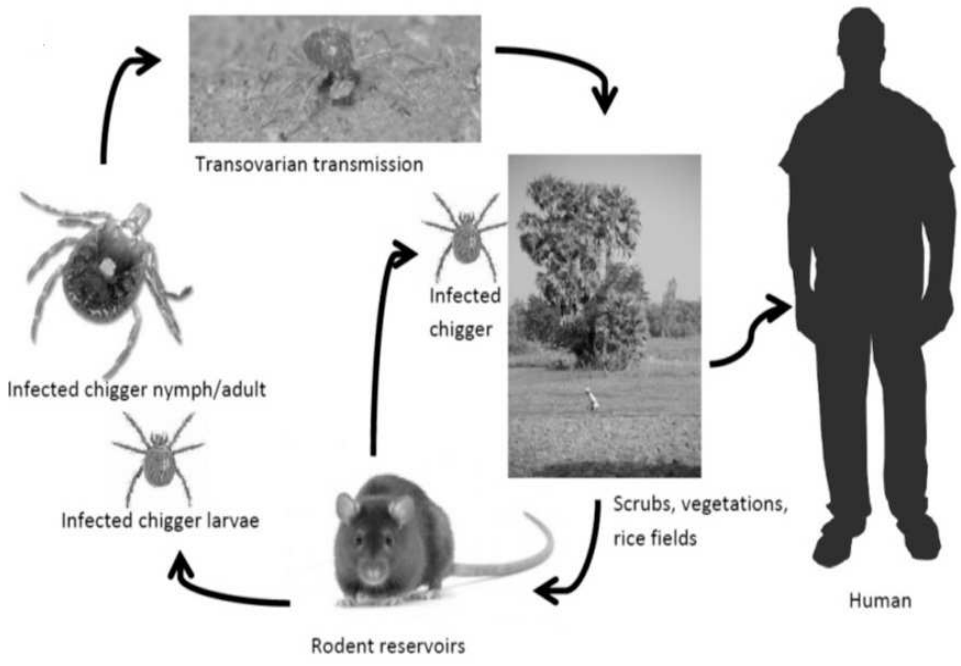


Fig-06: A simplified diagram showing life cycle of Orientia species adopted from Aung *et al, Am. J. Trop. Med. Hyg 2014* [57]

The chiggers feed usually on rodents and accidentally on humans, and transmit the infection during the prolonged feeding which can last for 1-3 days. Humans get infected during land clearing, logging, road building, and military operations.[152] Travelers who are involved with eco-tourism activities in rural areas are at high risk.[135,153-154] Incidence of scrub typhus is reported mainly in rural agricultural areas[149] and, some cases have also been reported in urban centers of Southeast Asia.[155] Geographically this disease is widely endemic in a confined area of the Asia- Pacific region, distributed in tsutsugamushi-triangle i.e. Afghanistan and India in the west; China, Japan, South Korea, and Taiwan in the north, and Australia and Indonesia in the south.[150]

Fig-07: Geographical distribution of tsutsugamushi disease adapted from Oaks *et al*.[156]

Scrub typhus is the most common rickettsial infection reported from India. After the initial frequent outbreaks during the Second World War, the incidence decreased greatly with the advent of antibiotics and wide use of insecticides. In India the first probable case of scrub typhus was reported from India- Burma border in 1943.[147] Further, serological evidence of scrub typhus cases has been reported from various states of India such as Himachal Pradesh,[21,151] Tamil Nadu,[22] Karnataka,[23,24,44] Kerala,[25] Rajasthan,[26] Manipur,[27] Pondicherry[28,29] Sikkim,[30] Punjab[157] Andhra Pradesh[158] Haryana[6] Jammu[159] Uttarakhand[160] and West Bengal.[161] Earliest reports of scrub typhus in India are from Tattersall et al.[162] Recent published reports have extensively studied the clinical and laboratory features with limited studies on the molecular epidemiology of the causative strains.[163-165] Scrub typhus is also reported as emerging and reemerging disease in some of the Indian states during the past decade.[171-174] Varghese *et al.* from Tamil

Nadu[22,163] and Mahajan *et al.* from Himachal Pradesh[21, 168,169] have reported most comprehensive data on scrub typhus and other rickettsial diseases in recent times.

Seasonal variations can be observed with scrub typhus in Southeast Asia. Incidence increases towards the end of the rainy season and the beginning of winter months (July–November).[23, 137]

3.4.1.6. Genotypes and serotypes of Orientia spp.

The prototype strains of *O. tsutsugamushi* are Karp, Kato and Gilliam which were described by Shishido *et al.* as early as 1969.[170] Newer serotypes namely Kuroki, Shimokoshi, Kawasaki, Boryong and others were added into the list over the time.[66,145] Typing of the newly isolated strains can be done using immunofluorescent (IF) testing using strain or type specific hyper immune sera or using monoclonal antibodies, however this method may not detect newer serotypes. With the advent of molecular techniques, strain typing is done by amplifying the *56-kDa* Type specific antigen (TSA) followed by Restriction Fragment Length Polymorphism (RFLP). More recently, sequencing and comparing the study sequencing results to the already available genotype sequence data in the databases and phylogenetic analysis is the common approach for genotyping. Genotyping have proved to be easier and efficient in typing the isolates over traditional serotyping.[143,175] Due to the significant molecular diversity in the strains of *O. tsutsugamushi*, until date more than 20 genotypes are identified.[143]

3.5. Rickettsial Vectors

Arthropods are the only known vectors of *Rickettsia*. All vectors of *Rickettsia spp.* Ticks, fleas, mites and lice are hematophagous ectoparasites and they act as both

reservoirs and amplifiers for most of the rickettsial spread throughout vertebrate and invertebrate populations. Maintenance of rickettsiae in an arthropod population occurs through transtadial and transovarial transmission.[38] Vertical transmission plays an important survival role when the vector population is low.[177]

3.5.1. Ticks

Ticks are obligate haematophagous arthropods that parasitize all classes of vertebrates and distributed worldwide. Recognized as human parasites for thousands of years, ticks have been described by ancient writers such as Homer and Aristotle. However, until the end of the 19th century it was not known that ticks were capable of disease transmission. After the mosquito, ticks are currently considered as the second most common vector of human infectious disease. Until the start of 20th century, it was not aware that ticks were associated with the transmission of bacterial diseases. Dutton and Todd demonstrated in 1905,[178] that tick relapsing fever was caused by *Borrelia duttonii* and transmitted by *Ornithodoros moubata,* and Ricketts *in* 1909 have shown that the wood tick *Dermacentor andersoni,* was involved in the transmission of *Rickettsia rickettsii*, the agent of Rocky Mountain Spotted Fever (RMSF).[35] Other common tick-borne bacterial diseases include *rickettsioses, ehrlichioses, anaplasmosis,* Lyme disease, some relapsing fever borrelioses (others are louse-borne), tularaemia and Q fever.[179]

Ixodidae, Argasidae and *Nuttalliellidae,* are the three major families of ticks identified so far. *Ixodidae* are also called as hard ticks due to the presence of sclerotized dorsal plate. They are most abundant and having wide range of distribution. *Ixodidae* family is further divided in to two groups they are Metastriata with 11 genera (e.g., *Hyalomma, Haemaphysalis, Rhipicephalus, Amblyomma and Dermacentor*) and the

Prostriata with only one genus *Ixodes*.[180] The members of *Ixodedae* family are responsible for the transmission of the SFGR and many other tick-borne pathogens. *Argasidae* or soft ticks has flexible cuticle, and have previously only been implicated with the non-pathogenic rickettsia *R. bellii*. Recent work had revealed the presence of SFG rickettsiae in soft ticks in Japan.[181] *Nuttalliellidae*, representing only one species, *Nuttalliella namaqua* distributed in eastern and southern Africa.[182]

Since the first report by Howard Ricketts, extensive studies have been conducted on the ticks *Dermacentor spp.* the vectors for *R. rickettsii* and, a considerable amount of information on tick-borne rickettsiae has been revealed.[35,52,183] The studies demonstrated that ticks acts as both vectors and reservoirs for *R. rickettsii*. Study of life cycle of Ixodid ticks shows how the maintenance and transmission of *R. species* occurs in the ticks population.

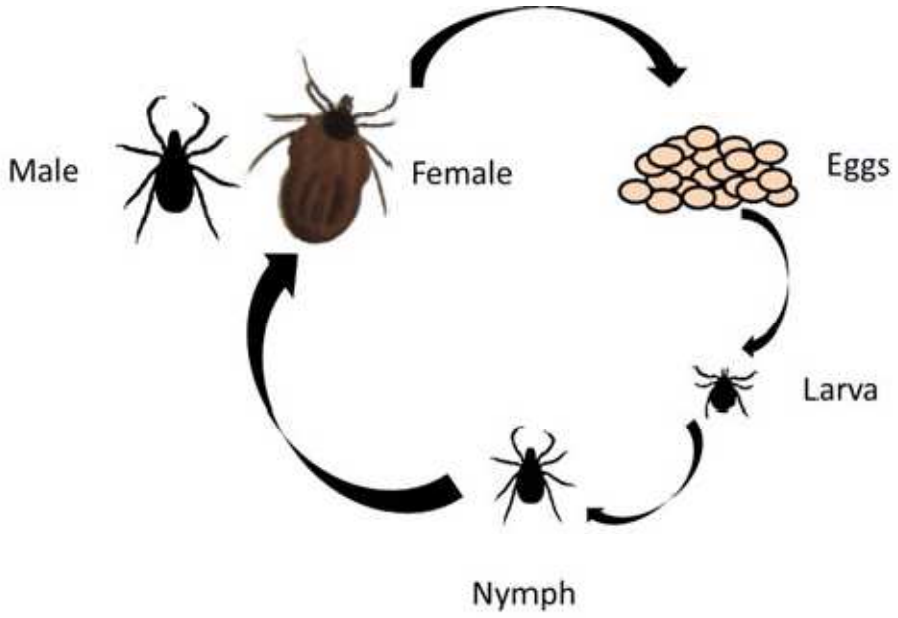


Fig-08. The four developmental stages of *Ixodidae* ticks adapted from Sonenshine and Roe [180]

The maintenance and transmission of *R. spp* in a tick population occurs by transovarial, transstadial, and horizontal transmission.[104,184] The *Rickettsia spp*. can be detected in many of the tick organs, the hemolymph, salivary glands and ovaries. The presence of rickettsiae in the salivary glands is essential for spread to hosts during feeding[185], and presence in the ovaries of adult females enables transovarial transmission of *Rickettsia*, from the female to her eggs. However, the percentage of eggs infected by transovarial transmission can vary. When the egg hatches into an infected larva, the tick will stay infected throughout its life. This mode of transmission of *Rickettsia spp*. from one life stage to the next, is called transstadial transmission.[186,187] Apart from transovarial and transstadial transmission, ticks can also get infected by pathogens from their blood meals. Horizontal transfer is when the host has an infection that the tick ingests through the blood,[188] but transmission can also take place during a blood meal on an uninfected host. This is called co-feeding transmission and occurs when two ticks feed in close proximity or subsequently to each other and transmit pathogens without any systemic infection of the host.[189] Sexual transmission of rickettsiae by ticks during mating has been observed however females infected by sexual transmission were unable to transmit the rickettsiae to their progeny.[190]

3.5.2. Fleas

Fleas are found predominantly on mammals and distributed worldwide. They act as important vectors for zoonotic diseases such as Plague caused by *Yersinia pestis*, Cat scratch disease caused by *Bartonella henselae* and bacillary angiomatosis caused by *Bartonella Quintana*.[132] The murine typhus caused by *Rickettsia typhi* and flea-borne

spotted fever caused by *Rickettsia felis* are the only two flea-borne rickettsial infections identified so far. Association of rat flea, *Xenopsylla cheopis* in the transmission of *Rickettsia typhi* was first described in 1926 by the American virologist Kenneth F. Maxcy.[191] Since then, *R. typhi* has been recovered worldwide in areas with rat infestation and in other flea species such as the cat flea *Ctenocephalides felis* and the mouse flea *Leptopsyllia segnis*. Transmission of R. felis to humans occurs through exposure to flea saliva, differing from *R. typhi* which has been found to be transmitted to humans through entry of flea faeces into the flea-bite site or skin abrasions, inhalation or the contamination of the conjunctiva.[132]

3.5.3. Lice

Lice are the well recognized host specific parasites found on humans and animals. The human body lice is specific only to humans, it cannot survive on any other animals. There are only three types of lice that live on humans, which are *Pediculus humanus capitis* (head louse), *Pediculus humanus corporis* (body louse, clothes louse), and *Pthirus pubis* ("crab" louse, pubic louse). Only the body louse is recognized as vector for the spread of disease in humans. Infestations of humans are known as pediculosis. The human body louse *Pediculus humanus* corporis is recognized as a vector for at least three diseases such as epidemic typhus caused by *Rickettsia prowazekii*, trench fever caused by *Bartonella quintana* and louse-borne relapsing fever caused by *Borrelia recurrentis*.[192] Various outbreaks and epidemics of the three diseases carried by the human body louse are evidence to its close co-evolution with humans. As a vector, human body louse was proved to be responsible for transmission of *R. prowazekii*, that caused outbreak of

typhus fever during the Napoleonic wars where 20% of Napoleon's troops died of typhus and caused death of 3 million Russians between 1917 and 1925.[193]

R. prowazekii is the only rickettsial species transmitted by the human body louse. The louse does not act as a reservoir for *R. prowazekii* and becomes infected as a result of feeding on a host with bacteraemia. Infected lice cannot survive more than 7 days due to the damage of epithelial cells caused by *R. prowazekii*.[194] Due to this lethal effect of *R. prowazekii* on its vector, the transovarial transmition of the causative organism does not occur in its vector population. In spite of this limitation, epidemic typhus cases are still being reported worldwide at the start of the 21st century, especially in the homeless and low-hygiene populations of developed countries. Factors contributing to the re-emerging threat of epidemic typhus are increasing incidences of human body louse infestations due to declining social structures and hygiene practices as a result of wars, economic instability and natural catastrophes.[195]

3.5.4. Mites

Mites are tiny arthropods belonging to the class Arachnida and are closely related to ticks. Though most species of mites are pests of agricultural crops, certain types of mites are proved to be parasitic on humans and spread disease such as Rickettsial pox caused by *Rickettsia akari*, the only one member of the genus *Rickettsia* which is known to be transmitted by the mouse mite *Liponyssoides sanguineus*.[196] However *R. akari* has also been isolated from other rodents in the U.S.A, Eurasia and Africa including the brown rat, gray dwarf hamster, golden spiny mouse, large-eared dormouse and bushy-tailed dipodil. [197] Many reports suggest that *L. sanguineus* is having a larger host range;

including larger vertebrate hosts have been supported with the discovery of antibodies against *R. akari* in dogs and cats in the U.S.A.[198]

The *Rickettsia akari* is maintained within *L. sanguineus* populations by transovarial transmission. The five life stages of *L. sanguineus* mites are; egg, larva, protonymph, deutonymph and adult.[199] For the moult and progress to the next stage, each life stage of mite requires a blood meal. With the multiple blood meals within its life cycle, the mite has the opportunity to pass on *R. akari* to infected vertebrate hosts several times.

3.5.5. Chiggers

Chiggers are the larval stage of mite *Leptotrombidium* belong to the order *Acarina* and family *Trombiculidae*. Chigger mites are recognized as the only vector responsible for the transmission of *Orientia spp.* which causes scrub typhus disease in humans. The most important chigger vector species in the Southeast Asia and in southern China is *L. deliense*, where as *L. pallidum, L. akamushi* and *L. scutellare* are the main vectors in Korea and Japan.[200] A newly described vector species *L. chiangraiensis* appear to be the predominant vector species in Thailand, found in cultivated rice fields.[146]

The chigger mite is very small (0.2-0.4 mm), is densely covered with hair. They can only be seen with the aid of microscope or magnifying lens. The mouth parts consist of a pair of chelicerae and a pair of palps, together giving the mite an appearance of having a false head. The chiggers are known to feed only once in their lifetime on mammalian tissue fluid,[49] and acts as the reservoir for the disease through transovarial transmission. Naturally, the mites found in nature and feed on diverse rodent population like interior of ear cups or on rumps of rats, mice, shrews, bandicoots and other small

mammals, reptiles and birds to maintain the mite life cycle. The rodents do not act as reservoirs; still they take on a key part in the population density of vectors. Humans are accidental and often terminal hosts of the diseases and are acquired when man enters the mite islands where the rodent chigger cycle is taking place.[146]

The vector has four main stages in its life cycle, namely eggs, larva, nymph and adult stage. The duration of the life cycle of the mite is greatly influenced by the temperature, in warmer climates it lasts for about 2-3 months and in cooler climates it can be as long as ≥ 8 months.[143] Only the larval stages are infective, as this is the only stage that feeds on mammals for body fluids which is necessary for its further maturation.

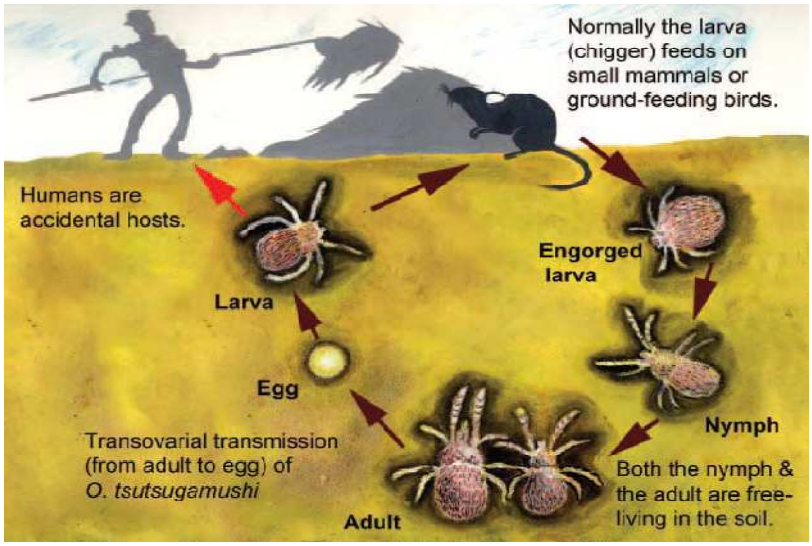


Fig-09: life cycle of a *Leptotrombidium* mite adapted from Jeong *et al*., 2007.[201]

The larvae is pale yellow to orange-red in colour with a circular body bearing three pairs of legs and branched hairs on the body and on the legs, a microscope is often required to visualize and confirm the chigger identity. They are seen in a cluster of 5 to 6 larvae on hosts. After the body fluid meal, the larvae detaches and drop to the ground and become nymphs, the scab formed at the detached site is an evidence of recent infestation.

Nymph stages are brick red in color and last for about 1-3 weeks and later mature to adults, which have four pairs of legs, the first pair being the largest of all. The life span of an adult is about 6 months. The adults and nymph stages are free living and feed on small invertebrates, their eggs and organic matter. The adult female mite lay eggs in a groups of several hundred on ground resulting in clumps of larval mites that can result in severe infestations of the host. Chiggers produce one generation each year, and are most abundant in the late summer and early autumn.[202] Once infected, the mite remains infected for its lifetime and the eggs can carry *O. tsutsugamushi* through transovarial transmission.

3.6. Antigenic structure of genus *Rickettsia* and *Orientia*

Rickettsia has species specific and group specific antigens. Spotted fever group *Rickettsia* has dominant outer membrane proteins *ompA* acting as an adhesion for host cells and *ompB* contains species specific epitopes, which is not found in typhus group rickettsia. Alkali stable polysaccharide antigens are found in some SFG *Rickettsia* and in some strains of typhus group.

O. tsutsugamushi possess an array of antigens, some used as the targets for molecular diagnosis and some to understand the evolutionary analysis.[203] The *sta*150, -110, -72, -58, -56, -49, -47, and -20 are important antigens of *O. tsutsugamushi*, cloned and expressed in *Escherichia coli*. The 56-kDa TSA is the most abundant antigen in *O. tsutsugamushi* and codes for an outer membrane protein and often recognized in humans infected with *Orientia*. The hypervariable regions in the 56-kDa TSA are considered to play an important role in the molecular typing of the stains (Figure 6.6.)[175]

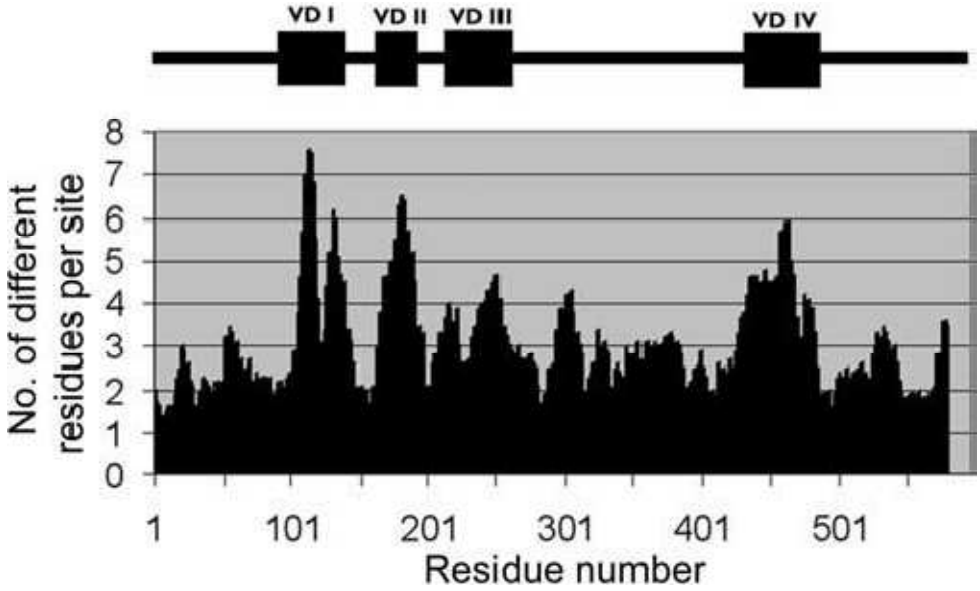


Fig-10. The diversity of amino acids in the alignment of the Hypervariable regions in the 56-kDa TSA, adapted from Kelly D. J *et al.*[143]

The plot illustrate the moving mean, for a gap of 10–amino acid residues, of the number of different amino acids observed at a position in the amino acid alignment (for >135 complete or nearly complete sequences from *Orientia* strains). The location of the highly variable protein domains VDI–VDIV within the amino acid alignment of the coding region is shown above the graph.

TSAs with length of 16-40 amino acids in the hydrophilic regions of the molecule are surface exposed and show different amino acid sequences among the strains.[175] All the variable domains are situated in hydrophilic regions. These VDs harbored type-specific differences, although single or multiple base substitutions or deletions of adjacent amino acid residues are recognized through the antigen. The alignment studies highlighted both diversity and similarity among strains, in VD regions the similarities in the amino acid sequences between individual strains vary significantly.

VD I was located to amino acids 105-132, nearly consisting of 22-28 amino acids, VD II was located to amino acids 151-176, consisting of 16-26 amino acids, VD III was located to amino acids 202-246, consisting of 24-35 amino acids and VD IV is the largest domain located to amino acids 402-451 consisting of 34-40 amino acids.[204] The amino acid sequences shows varying homology between strains, i.e highest similarity of 94.1% at VD IV region between Gilliam, Karp, and Kuroki strains to lowest similarity of 51.5% between Kato and Gilliam strains at VD III region with two to four amino acid substitutions. High conservation is seen in the amino acid sequences at VDIII region between the Gilliam and Kawasaki strains and between the Karp and Kuroki strains. However, the sequences of the other strains show greater variation in each VD region.[175]

Many of the monoclonal antibodies raised against *O. tsutsugamushi* isolates have been found to react only with the corresponding homologues strain but not with heterologous strains indicating that this polypeptide has strain-specific epitopes. This property of strain-specific monoclonal antibodies directed against this polypeptide has been demonstrated by researchers to characterize antigenic variants of *O. tsutsugamushi* isolated from various sources across the world.

Some of the Monoclonal antibodies raised against TSA also showed cross reactivity with some or all heterologous strains indicating that this polypeptide consists of both strain-specific and common epitopes. Human IgM predominantly bound to the antigenic domain I (AD I; amino acids 19–113) and antigenic domain III (AD III; amino acids 243-328), whereas Human IgG showed preferential binding to AD I.[205] These results suggested that AD I, AD II, and AD III are useful in the induction of humoral immunity against *O. tsutsugamushi*.

The amino acid region encompassing residues 131-201 (containing ADII and VDII) from Karp, Kato and Boryong strains were reactive with both homotypic and heterotypic antibodies. Lacking of the antibody specificity, and thus the ADII region was suggested to be dispensed and unsuitable for vaccine development.[206]

3.7. Pathogenesis

Rickettsial disease or rickettsioses are caused by known pathogenic bacterium from the genus *Rickettsia* and *Orientia*. Although all species of *Rickettsia* differ in transmission, host- pathogen interactions and pathology, most of them exhibit similarity in pathogenesis.[167] All pathogenic rickettsiae need arthropod vectors for transmission; but, how the pathogen transmitted from the arthropod to humans can differ. The actual mechanism by which the rickettsiae spread from skin at the site of arthropod bite to internal organs is still uncertain. The most probable routes may be through the blood stream or lymphatics. Tick and mite borne rickettsiae are transferred to the host by the tick or mite saliva through bite site during feeding. Cases of transmission of rickettsiae through the conjunctiva have occurred with exposure of infected tick haemolymph on fingers after crushing an infected tick. Flea and louse borne rickettsiae are transmitted by faeces that enter the host via bite site or cuts on the skin.

Although target cells at the site of inoculation are unclear, the recent studies demonstrated that rickettsiae perhaps infect dermal cells such as vascular endothelium mainly and, fibroblasts, lymphatic endothelium and macrophages.[45,207] Once rickettsiae have penetrated and initiated infection, spreading throughout the host is facilitated significantly by the lymphatic and vascular systems though the action of rickettsial

adhesions acting on specific receptors on host cells.[208-209] In case of scrub typhus, it is believed that the *O. tsutsugamushi* which may be present in the salivary glands of infected chiggers is introduced into the dermis during the body fluid meal and subsequently spread to the regional lymph nodes and blood stream. [210] The hypothesis of antigenic spread via the lymphatic system is supported by the occurrence of lymphadenopathy in the same region as the site of inoculation.

Recently with the introduction of new culture techniques and imagery technology, interactions between the rickettsiae and their host are well demonstrated. Severity of rickettsial disease can be correlated to patient age as the main consistent factor observed in almost all cases. Initial hypothesis for rickettsioses having a higher fatality in males (esp. for Rocky Mountain spotted fever) has been disproved by recent studies.[113] Other factors affecting rickettsiae-host interaction would be underlying patient diseases and enhanced oxidative stress increasing the severity of illness. Mediterranean spotted fever shows greater virulence in patients with diabetes mellitus.[209]

In SFGR, pathogen attach and enter the host cell receptors via two surface proteins, *ompA* and *ompB* with host cell protein Ku70.[45] Adhesions of outer membrane protein B (*ompB*) with Ku70 result in a cascade reaction which involves the migration of more Ku70 and ubiquitin ligase to the binding site and initiates internalization of adherent rickettsia into the host cell by activating clatherin- and caveolinmediated endocytosis, and rearranging of the actin cytoskeleton.[211] Sca1 and Sca2 of the SFG had recently been reported to promote adhesion to mammalian cells.[212,213]

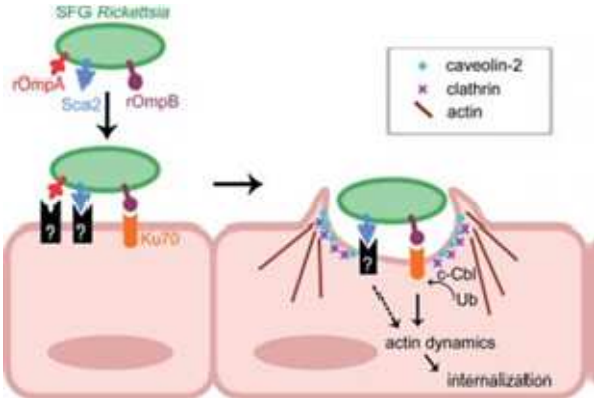


Fig-11: Adherence and initiation of internalization of Rickettsia by host cell adapted from Cardwell & Martinez, Infection and Immunity 2009. [213]

Rickettsial infections are mainly vasculotrophic in nature and present hypothesis about immunopathogenic mechanisms concerns that of oxidative stress, leading to endothelial cell injury, increased micro vascular permeability, and possibly development of a procoagulant state. Once inside the cell, rickettsia quickly escapes the phagosome into the cytosol by digesting the phagosomal membrane via the action of the enzymes phospholipase D and hemolysin C (TlyC), [45,214] and when free inside the cytoplasm of the host cell, the bacteria can make use of the cell's nutrients and machinery to grow and spread.[215] *Rickettsia* infected endothelium cells produces the proinflammatory cytokines interleukin-6 (IL-6), IL-8, and monocyte chemoattractant protein-1.[45] Mechanism of intra and intercellular spread of rickettsiae is facilitated by actin polymerization, shown to be present in all spotted fever group rickettsiae except *R. peacockii*. Activation of the actin polymerization occurs at one pole of the bacterium, and the continuous conversion of globular actin to filamentous actin propels the rickettsia through the cytosol eventually hitting the host cell membrane.[216,217] The mechanisms through which rickettsia polymerizes actin are, one early in the initial infection phase, which produces shorter

curved actin tails (probably for intracellular movement) where the *RickA* protein recruits and activates the host cell's actin-related protein (Arp) 2/3 complex, which in turn initiates polymerization of actin. Later in the infection, after rickettsial replication, there is a formation of longer and straighter actin tails (probably for intercellular movement) governed by the Sca2 protein through an unknown pathway.[217] In comparison, *R. prowazekii* and *R. typhi* of typhus group, which has a disrupted *RickA* gene and is unable to polymerize actin, multiplies exponentially until the host cell burst, causing necrosis, and then infects new host cells.

Clinical severity is determined by the host immune responses, and other clinical conditions such as older age, glucose-6-phosphate dehydrogenase deficiency, diabetes.[47] The most common complications caused by rickettsial infections are due to the damage and dysfunction of the vascular endothelial layer, which leads to vasculitis and may cause CNS complications, respiratory and multi-organ failure.[215] Cytotoxic CD8+ T cells plays major role in clearance of acute rickettsial infections. CD4+ and CD8+ T cells facilitate differentiation of B lymphocytes to plasma cells and produce antibodies against *ompA* and *ompB* to provide protection against reinfection.[218] Production of antibodies against rickettsial lipopolysaccharides do occur, but they do not appear to provide any protection.[219]

In case of scrub typhus, it was initially believed that the disease spreads through the lymphatic system, however, Walsh *et al.* in 2001 demonstrated the pathogen in the mononuclear white blood cells in acute cases of scrub typhus suggesting the possibility of direct blood borne spread.[220] Notably, in an *in vitro* study from Laos showed that scrub typhus infection was associated with production of more prominent levels of

inflammatory cytokines and pronounced activation of coagulation pathways when compared with murine typhus.[152] Further studies have demonstrated that scrub typhus infection induces type 1 immune response and results in the increase of levels of interferon-γ, tumor necrosis factor-α, IL-12p40, IL-18, and IL-15.[221] These intrinsic differences in host responses elucidate why scrub typhus infection produce more severe disease and disturbed biochemical parameters compared with murine typhus or SFGR.

Host immune system plays an important role in controlling scrub typhus, the acute self-limiting febrile illness caused by *O. tsutsugamushi.*[222] Laboratory based experimental evidence on mice and circumstantial evidence from naturally acquired human infections suggested that relapse rates reduced after the withdrawal of chloramphenicol therapy. These studies suggest that a protective immune response was developed in these cases.[146,223] The actual mechanism by which the *O. tsutsugamushi* cleared from blood is not completely understood. Anti *Orientia* antibodies and cell mediated cytotoxic mechanism are known to enhance the clearing of *O. tsutsugamushi* from blood. Infection with *O. tsutsugamushi* does not lead to long term immunity and lead to frequent re infections. The protective immunity lasts for only one to three month for a heterologous serotype, whereas it can last for up to one to three years in case of homologous serotypes. Often the first infections are symptomatic than subsequent infections and reinfection due to a heterologous strain produce milder disease. Variations in the severity of the diseases has been observed with the prototype strains of *O. tsutsugamushi.*[143]

3.8. Clinical Features

Clinically, the same rickettsial species can cause different symptoms from case to case, and different species can cause the same complex of symptoms.[224] Early signs and symptoms of rickettsial infections are nonspecific and may vary from mild to severe. Unless there is a high index of suspicion, it is likely to be missed as the clinical presentation may resemble other common viral infections in the tropics, making clinical diagnosis more difficult.[1,45]

Incubation period of each rickettsial infection is different and it varies between 2-21 days. Clinical presentations may differ with the etiological agent and patient; however, most common clinical symptoms that typically develop during initial stages of rickettsial infection include acute fever, headache, myalgia, nausea or vomiting, generalized lymphadenopathy and hepatosplenomegaly, which are nonspecific and seen common in several other febrile diseases also. The two characteristic signs that usually differentiate rickettsioses from other febrile illness are the presence of rashes on various parts of the body and an inoculation eschar at the tick or mite bite spot. However, there are several reports of "spotless" spotted fever and "escharless" rickettsioses too.[1,72,226] Major symptoms are similar for most rickettsial diseases, variations that do occur can vary greatly from classic symptoms.

Clinical manifestations of rickettsial infections are detailed herein;

3.8.1. Fever

Acute fever is the most common symptom of the rickettsial disease. Sudden onset of fever, sometimes high-grade, with chills, occasionally with morning remissions associated with headache and myalgia should always be considered for clinical diagnosis

of rickettsial disease particularly in endemic region with history of tick bite, contact with dogs, history of residence or recent visit to disease hot spots or entry to natural habitat of ticks and mites. In a study by Somashekar *et al*. from India, among 180 children admitted with acute febrile illness excluding the cases with other common causes for fever, 24% were clinically and serologically confirmed to have rickettsial infections. Among positive cases, Scrub typhus formed the largest group (62.8%) followed by spotted fever (32.6%) and endemic typhus fever (4.7%).[227]

3.8.2. Headache and Myalgia

Severe frontal headache and generalized myalgia especially in muscles of the lumber region, thigh and calf is seen in variable proportion of patients. In young children headache is noted less frequently than in adults, but when it occurs, it is often stubborn to therapy.[228]

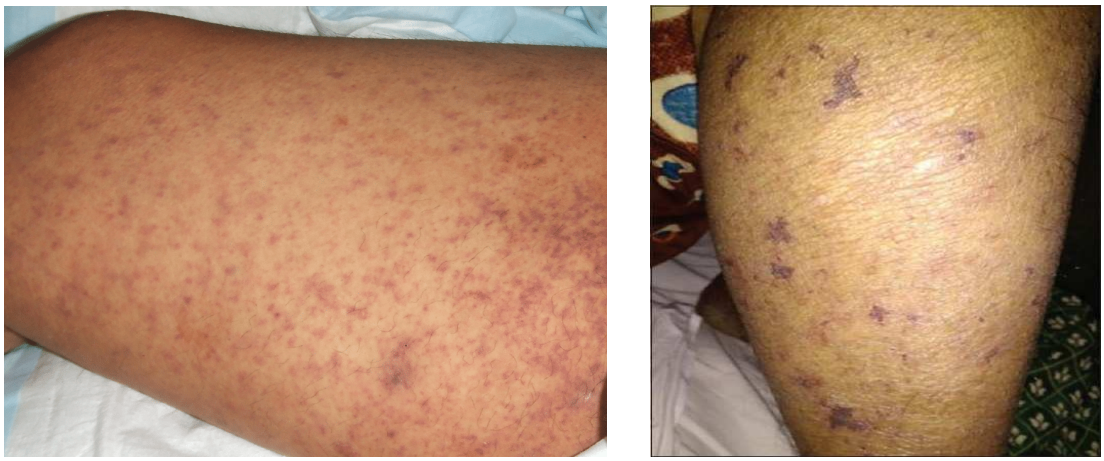
3.8.3. Lymphadenopathy

Regional or generalized lymphadenopathy associated with eschar is seen mostly in scrub typhus patients.[229] Tickborne lymphadenopathy (TIBOLA) and Dermacentor-borne-necrosis-erythema-lymphadenopathy (DEBONEL) are seen in some of the new or emerging rickettsial infections caused by *Rickettsia slovaca* and *Rickettsia raoultii*.[2]

3.8.4. Rash

Rash is considered as major sign of rickettsial disease, though it is neither appear nor in all the patients.[3,45] Thus it should be remembered that in some cases spotted fevers could be spotless too! Presence of rash is common in spotted fever and is very rare in scrub typhus. Rash usually becomes visible after 3-5 days of onset of symptoms. Initially rash appears in the form of pink, blanching, distinct macules which subsequently becomes

maculopapular, petechial or hemorrhagic (Figure-12). Sometimes palpable purpura, a typical sign of vasculitis is seen. Occasionally petechiae expand to ecchymosis and gangrenous patches may develop. In a very few cases, gangrene of digits, earlobes, scrotum, nose or limbs may also occur secondary to vasculitis and thrombosis. Initially rash appears near ankles, lower legs and wrists. There after it spreads centripetally to involve whole body. Though presence of rash on palms and soles, considered so typical symptom of rickettsial disease, it can be seen in other diseases also like infective endocarditis, syphilis, meningococcemia, enteroviral diseases and adverse drug reactions. The appearance of rash with typhus group rickettsioses is quite atypical, initially appears on trunk, spread centrifugally and generally sparing palms and soles.

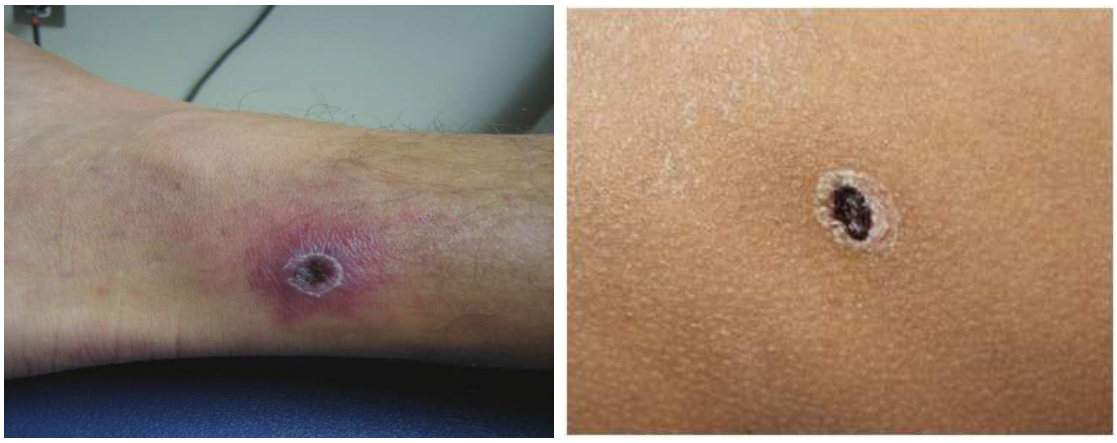


A **B**

Fig-12: Appearance of Rashes: Petechial rash on thigh region of a patient with Rocky Mountain spotted fever caused by *Rickettsia rickettsii* (A)adapted from Parola *et al.* [70] and, Retiform purpura over leg caused by Indian tick tphus (B) adapted from Manjunath H *et al.* 2017.[13]

3.8.5. Eschar

A necrotic eschar at the site of tick/mite bite is seen in variable proportion of patients with rickettsial infections such as Scrub typhus, Indian tick typhus and Rickettsialpox.[229]. Generally eschar is painless; lesion may extend upto 1 cm in width with a black necrotic centre resembling the mark of a cigarette burn, which is surrounded by an erythematous rim.(Figure-13)



A B

Fig-13: Necrotizing eschar with erythematous halo over the right leg of a patient with the Rickettsial infection caused by *R. parkari* (A) adapted from Todd Meyers *et al.* 2013,[231] and Left infra axillary region of patient with Scrub typhus infection (B) adapted from Tony Ete *et al.* 2016.[232]

Tipically, the eschar develops during the 6- 21 days incubation period, and by the time of onset of fever the eschar would be well developed. Eschar is a characteristic sign of scrub typhus infection with a varying frequency of 7-97 per cent.[233] Typically a single eschar is found on the neck, axillae, chest, abdomen and groin, but multiple eschars have also been documented.[234](Figure-14) Eschar on axilla, scrotum, perianal region may be missed if not looked into carefully because these may lack the black scab, and appear as shallow yellow based ulcers without surrounding hyperemia.

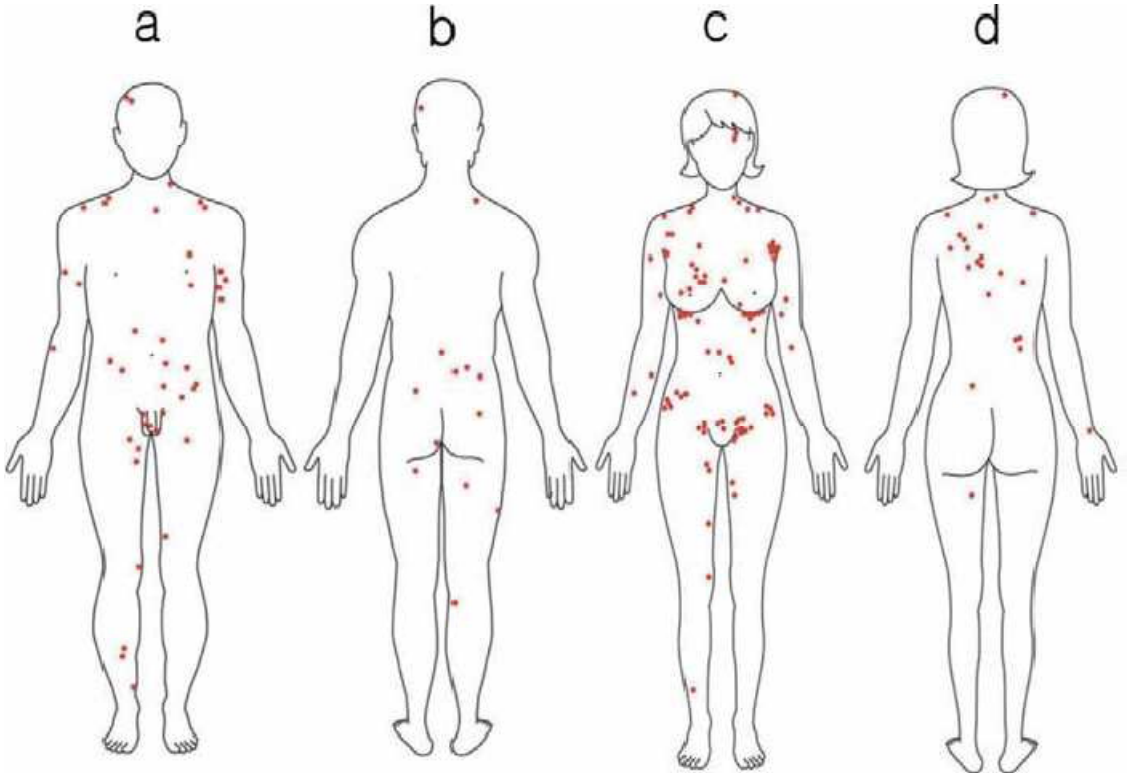


Fig-14: Probable distribution of eschar on various parts of the body in scrub typhus patients adapted from Kim D.M *et al*., 2007. [49] (a) male front (b) male back (c) female front (d) female back.

3.8.6. Systemic features

Gastrointestinal symptoms including nausea, vomiting, abdominal pain and diarrhea are seen in most of the rickettsiosis cases with varying frequency. Constipation is seen mostly in epidemic typhus. Respiratory symptoms include cough and distress are sometimes seen. Neurological symptoms like dizziness, drowsiness, disorientation, tinnitus, photophobia, delirium, meningismus and visual disturbances; are seen more commonly with typhus group rickettsioses. The word 'typhus' refers to cloudy state of consciousness ('typhos': cloud or smoke). Other symptoms like Periorbital edema,

conjunctival hyperemia, epistaxis, acute reversible hearing loss and arthralgia are sometimes reported.

3.8.7. Complications

Rickettsial infections sometimes create severe life threatening conditions and show a fulminant course. The severity of rickettsial disease has been associated with differences in virulence nature of pathogen and host- related factors such as age, comorbidities, delayed diagnosis, immunosuppression, hepatic and renal dysfunction, CNS and lung involvement.[207] Rickettsial infections, particularly spotted fever group is reported show fulminant course in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency[241].

3.8.7.1. Scrub typhus:

The complications of scrub typhus typically develop during second week of illness. Common complications include bronchopneumonia, thrombocytopenia, toxic hepatitis and acute cholecystitis.[235] Severe complications include acute respiratory distress syndrome (ARDS), multiorgan dysfunction syndrome (MODS), renal failure, myocarditis, meningoencephalitis and septic shock leading to death.[168] Isolated cases of acute encephalitis, abducens nerve palsy, and acute transverse myelitis due to *O. tsutsugamushi* were also reported. [236-237]

3.8.7.2. SFG Rickettsiae

Severe complications of SFG include superimposed bronchopneumonia, congestive heart failure (caused by fluid overload)skin necrosis and gangrene, acute renal failure, and neurological manifestations, including confusion and hearing loss, bilateral pulmonary

infiltrates, acidosis, abnormal liver function indices, thrombocytopenia and hypoprothrombinemia [207, 237]

3.8.7.3. Typhus group rickettsiae

Meningoencephalitis may occur in 50% with meningism, tinnitus, hyperacusis followed by dysphoria, deafness, dysphagia, agitated delirium and sometimes coma. Survivors may suffer transverse myelitis, hemiparesis, peripheral neuropathy with hyperesthesia and prolonged psychiatric disturbances. Secondary infection leading to gangrene, myopericarditis, pneumonia, and pleurisy bronchopneumonia, otitis media, parotitis and peripheral blood vessels blockage may occur. [150,238,239]

3.9. Mortality in Rickettsiosis

Most of the rickettsial diseases are reported to cause moderate illness in humans. However, Rocky Mountain spotted fever, Mediterranean spotted fever, epidemic typhus and scrub typhus are known to cause severe complications, and may become fatal in 20-60% of untreated cases. Due to non specific manifestations, rickettsial disease may be misdiagnosed as other common febrile illness, even by clinicians experienced with these diseases.[147,240]

In SFG rickettsial infections, the overall mortality rate reported without antibiotic therapy is approximately 25%; however, the mortality rates are seen higher in elderly persons and people with glucose-6-phosphate dehydrogenase (G-6-PD) deficiency.[2, 241] In the United States, tickborne rickettsial diseases continue to cause severe illness and death in otherwise healthy adults and children, in spite of the availability of effective antibacterial therapy. Lack of rash or late- appearance of rash in RMSF has been

associated with delays in diagnosis and increased mortality. Overall mortality rate reported from United States is 5-7%.[242,243] In a study from Brazil, reported highest mortality of 55% during the period of 2007-2015 which could be related to diagnostic difficulties and lack of prompt treatment.[244] In a report by N.B. Rathi *et al*. from central India, the mortality rate with SFG rickettsiae cases was 9%.[237] The main reason for the fatal outcome are delay in diagnosis and inappropriate treatment. Solid immunity usually follows recovery from SFGR. Rickettsialpox is normally self-limiting and deaths have not been reported. Boutonneuse fever is traditionally characterized as a benign rickettsial infection, very rarely, rapidly fatal course is reported in unhealthy children.[2]

The mortality rate in untreated epidemic typhus cases associate with the patient's age. Mortality may be unusual in children younger than 12 years, but rates increase to as high as 60-70% in individuals aged 50 years and more.[2] Most patients who recover from the disease develop immunity. Murine typhus, although it is considered to develop a mild illness, may cause mortal or severe illness if misdiagnosed or inadequately treated. Reported mortality ranges from 1% to 4%, and higher mortality is associated with lack of antibiotic therapy.[2,129]

In Scrub typhus cases, fatalities are rare with use of antibiotics. In the pre antibiotic era, the fatality rate of Scrub typhus cases was ranged from 35% - 50%.[143,147] From India the earliest report of mortality due to scrub typhus is from India- Myanmar border with a mortality of 5% seen during the Second World War.[116,143,245] In a study by Kumar K *et al.* from Himachalapradesh, they have reported a case fatality of 17.3%.[246] Mathai *et al.* from Tamil Nadu have reported a mortality rate of 11% during their study on outbreak of scrub typhus during the period of October 2001 to February 2002.[247]

Frequent reinfections is observed in the patients due to the heterogeneity of scrub typhus strains. Mortality from this disease is 7-30%.[2] Some other cases reports have shown overall mortality rates for scrub typhus range from 2.6% to 15%.[46,137]

3.10. Diagnosis of Rickettsial Infections

Currently, the diagnosis of rickettsial infections is done considering several aspects: the clinical symptoms, epidemiological information and laboratory findings. Before the introduction of laboratory diagnostic methods, identification of the infections and patient management was done only on the basis of clinical presentation and knowledge of clinicians on prevalent rickettsial diseases in their locality.

3.10.1. Clinical Diagnosis:

The most important clinical diagnostic clue for rickettsial infection is the presence of rash on the body and an eschar at the site of tick or mite bite, but it usually requires 5-7days to develop and may not appear or seen in some of the cases. Without specific diagnostic tools, the diagnosis of rickettsial infections is really challenging as most of the common clinical symptoms of rickettsial disease like fever, myalgia, headache and local lymphadenopathy are not specific to rickettsial disease and may be seen in a number of other infectious diseases like malaria, measles, meningococcaemia, typhoid fever, influenza, leptospirosis etc.

History of residence or recent visit to the areas where the rickettsial disease are prevalent (disease hot spots), entry to the mite islands, exposure to ticks or evidences of tick, mite bites in a febrile patient accompanying with common clinical manifestations are the major indication of probable rickettsial infection and may aid in early selection of specific treatment.[33,57,243] Febrile patients from disease endemic areas must be vigilantly

searched for the presence of an eschar that would give substantial evidence to initiate anti-rickettsial treatment.[48]

Leukopenia with marked left shift, mild hyponatremia, thrombocytopenia and mildly elevated hepatic transaminases levels are common and particularly practical clinical laboratory features suggestive of rickettsial disease, although the absence of these features does not rule out diagnosis of rickettsial infections.[1]

3.10.2. Laboratory Diagnosis

Currently, laboratory diagnosis of rickettsial infections is done by two methods; indirect method and direct method. Indirect method refers to the demonstration antibodies to rickettsial antigens by several serological assays. In direct methods, the pathogen present in the blood stream, tissues and eschar are detected directly by molecular diagnosis and culture.

3.10.2.1. Serological Methods

Generally diagnoses of Rickettsial infections are largely based on various serological methods in most of the laboratories.[57] Weil felix test, Enzyme-linked immunosorbent assay (ELISA), Immunofluorescence assay (IFA), Western Blot, Complement fixation, Indirect hemagglutination and Latex fixation tests are the documented serological assays that demonstrate antibodies to rickettsial antigens. As detectable antibody titres usually take 5 to 10 days to develop, serological methods may not be so useful for diagnosing acute illness, however, helpful in the confirmation of rickettsiosis during the later stages.[87] Several newer serology based methods with good

diagnostic potential have been described over the years, however even today Weil Felix test the only available method in many of the rural tropics.

3.10.2.1.1. Weil Felix Test:

Weil felix test is one of the oldest serological test used for the diagnosis of rickettsial infection. It was first described in 1916 by Edmund Weil and Arthur Felix on the basis of their observation that serum of patients infected with epidemic typhus was found agglutinating in the presence of some of the non rickettsial bacteria belonging to the genus *Proteus spp.*[50] Further research on such a phenomenon found that the cause was due to somatic (O) antigen sharing between the two genera. The basic principle of Weil-Felix test detects antibodies to various Proteus antigens that cross-react with rickettsiae except *R. akari*. [87,249] Whole cells preparations of *P. vulgaris* OX-2 react strongly with sera from SFG positive patients except sera from RMSF positive cases. Sera from typhus group rickettsiae positive patients as well as RMSF positive patients react with whole cells of *P. vulgaris* OX-19. Subsequently, whole cell preparations of the *Proteus mirabilis* OX-K strain was found to agglutinate with sera from scrub typhus positive patients and later used in the diagnosis of *O. tsutsugamushi*- related infections. [48,250] However, WF test is not applicable to diagnose the patients with Brill-Zinsser disease or infected with *R. akari* due to the inability of the antibodies to agglutinate with the whole cell preparation of *Proteus* starins.[87]

By the Weil-Felix test, agglutinating antibodies mainly IgM are detectable only after a week (5 to 10 days) of acquiring infection.[87,251] Conversely, the test may be found positive without rising IgM antibody titers also.[87]

WF test is based on the principle of agglutination and which is visualized macroscopically. Test is easy to perform, economic, commercially available and remains the first line test in most of the rural tropics of Asia. The test can be performed both in a slide and tube format with the latter being more sensitive.

False positive reaction could be seen with dengue, leptospirosis, louse borne relapsing fever, and as well with *Proteus* urinary tract infections.[252] The poor performance characteristics of the Weil-Felix test is now well known for the diagnosis of MSF,[254] RMSF,[65,253] murine typhus, epidemic typhus[255] and scrub typhus.[256] The other difficulty of the test is related to the cut off titers for the assay. Hence, for deciding the cut-off titers, endemic titers must be considered. Titers of 1:80 or 1:160 are the commonly reported cut off titers for WF test in Indian population.[44,246,257]

3.10.2.1.2. Immunofluorescent assay (IFA)

The immunofluorescence method was first proposed by Woodward *et al.* in 1976.[258] Philip, in 1978 recommended the use of immunofluorescence assays (IFA) for the diagnosis of rickettsial infection using serology as a replacement to the Weil-Felix method.[259] IFA is the widely accepted reference standard/gold standard method currently for the diagnosis of rickettsial diseases and is used frequently in providing rapid diagnosis to suspected rickettsial infections.[226,239,259] The main advantage of IFA in comparison to other previously described serological assays is that it can simultaneously detect antibodies to up to 9 antigens in a single drop of serum.[260] In IFA one can detect both IgM and IgG antibodies separately or together.

The identification of specific IgM antibodies by IFA found to provide strong evidence of recent active infection caused by various rickettsial species, even though the

diagnosis may be masked by a prozone phenomenon.[261] In addition, this technique is affected by rheumatoid factor, thus requiring the use of a rheumatoid factor absorbent before IgM detection. The persistence of antibodies in patients with scrub typhus infection remains contentious because old reports have demonstrated the antibodies persistence over a period of many years,[262] whereas more recent studies in last decade have confirmed an annual reversion rate from titers of greater than 1:50 to titers of less than 1:50 in 61% of subjects.[263]

By IFA a rickettsiosis is judged as ongoing or recent if the antibody titre shows a fourfold increase from the acute-sera (taken during 0-14 days of illness) to the convalescent (follow-up) sera taken 2-6 weeks after disease onset.

IFA is highly sensitive but it faces the difficulty of the lack of intra-rickettsial group specificity. IFA is proved to be fast and accurate for the diagnosis of rickettsiosis, but differentiation between species of rickettsiae as the cause of infection is often impossible due to cross reacting antibodies.[264] However, using multiple antigens and comparing the titres can provide guidance in identifying which species a patient has the strongest reaction to.[265] The cut off titers in various studies varied significantly among geographical areas and it can differ between IgG and IgM depending on species. Cut off titers for IFA ranged from 1:10 to 1:400, and is usually set between 1:32 and 1:128.[266]

On the contrary, the IFA test is expensive, requires a fluorescence microscope, and also require trained personnel to perform the test.[44] Preparation of antigen slides is a complex process and requires expertise and BSL3 level lab to mass-produce the cell cultures to coat on M-IFA slides. Currently M-IFA kits are commercially available and

some of the sources are Fuller labs, California, USA and Australian Rickettsial Reference Laboratory (ARRL), Victoria, Australia.

3.10.2.1.3. Complement fixation (CF) test

With the development of techniques for detection of increasing rickettsiae, the complement fixation (CF) test was adapted for the detection of specific antibodies to rickettsial organisms. Washed particulate rickettsial antigens used in CF test are species specific for the typhus group and SFG rickettsiae, however, cross-reacting antibodies among groups are also observed.[267] The CF test is strain specific for *O. tsutsugamushi*. A significant CF titer is obtained only with the homologous antigen of the strain that has caused the infection. This specificity, particularly seen with sera collected at acute-phase of infection, implies that all strains known to be widespread in a region must be incorporated to ensure the detection of every positive serum specimen.[268] Antibody titers observed in the CF test demonstrate better association with IgG titers than with IgM titers obtained by IFA. Results may vary according to the method of antigen production and the amount of antigen used in the assay.[267] The use of 8U of antigen increases the sensitivity of detection of the early IgM antibody response, but also increases the numbers of cross-reactions between antibodies to typhus group and SFG rickettsiae.[267] Due to its practical difficulties in preparation and standardizing the antigens for the assay and lesser sensitivity and specificity the test was not routinely used for the diagnosis of scrub typhus disease.[252]

3.10.2.1.4. Enzyme linked Immunosorbent assay (ELISA)

The Enzyme-linked immunosorbent assay, like IFA, detects the binding of specific antibodies in a serum sample to an antigen. The ELISA was reported to have

similar sensitivity and specificity in comparison to the current gold standard test, the Immunofluorescence assay (IFA). In 1977, for the first time ELISA method was introduced for the detection of antibodies against Rickettsia prowazekii and Rickettsia *typhi*.[269] ELISA was found to be highly sensitive and reproducible, allowing the detection of IgM and IgG antibodies. This method was later adapted for the serodiagnosis of other rickettsial infections like RMSF[270] and scrub typhus.[252] In an original approach, a "paper ELISA," was proposed was proposed by Crum W H *et al.*, for the detection of anti- *O. tsutsugamushi* antibodies.[271] Initial steps of this method are similar to those followed in IFA, but an anti-human IgG peroxidase conjugate and substrate are saturated on a filter paper, to visualize the reaction. In addition, a modified inhibition ELISA method has also been demonstrated for the use in serodiagnosis of scrub typhus infection caused by *O. tsutsugamushi* Kawasaki strain.[272] In this method monoclonal antibodies were coated in the wells and inhibition of antigen absorption was evaluated by mixing test sera and crude antigen. It was estimated that the assay is more sensitive than IFA, especially at the initial stage of the illness.[272] The most recent, promising and interesting approach is the modified ELISA called as Aptamer based ELISA type assay (ALISA).[273] Most of the ELISA kits are now commercially available and a general cut off titer is provided with the kit literature. However, it is always suggested to determine the cut off titers in local population. Currently recombinant antigens are most commonly used in place of homologous antigens of *Rickettsia* and *Orientia*.[274]

3.10.2.1.5. Microagglutination test

The microagglutination test is based on the detection of interactions between antibodies and whole rickettsial cells.[275] This method has not been widely used as the test require a large amount of purified rickettsial antigen, and non availability of these antigens commercially.

3.10.2.1.6. Indirect haemagglutination test

In indirect hemagglutination (IHA) test, antibodies are detected using glutaraldehyde-stabilized erythrocytes treated with a rickettsial erythrocyte-sensitizing substance obtained from Rickettsial species. It exhibits group specificity, but the cross-reactivity among the species of RMSF, rickettsial pox, and MSF rickettsiae is observed. This test detects both IgM and IgG antibodies, but agglutination is seen more efficient with IgM antibodies.[276] In comparison with the IFA, the IHA procedure give lower titers but shows comparable detection of seroconversion with most paired sera. The IHA test demonstrates significantly higher titers than the complement fixation test and is more sensitive than either the complement fixation or Weil-Felix test in identifying seroconversion. No agglutination occurs when sensitized erythrocytes tested with rodent sera known to contain rickettsial antibodies. [276]

3.10.2.1.7. Latex agglutination test

In the latex agglutination test, an erythrocyte-sensitizing substance is used to coat latex beads.[277] The reactivity exhibited by this test is not exactly the same as that of the indirect hemagglutination test, as the ESS on latex beads perhaps contains more antigenic fractions than the ESS adsorbed onto erythrocytes. This test is rapid (15 min) and does not require complex instrumentation. Latex agglutination test shows reaction with both

IgM and IgG antibodies, but the agglutination efficiency of this test is greater when the antirickettsial IgM/IgG ratio is $\geq$1. This test allows the demonstration of antibodies within one week after the onset of illness. Significant antibody titers disappear after two months.[277]

3.10.2.1.8. Immunoperoxidase assay

As an alternative test to IFA, an immunoperoxidase assay has been developed for the diagnosis of scrub typhus. [278] Later the test was evaluated for the use in the diagnosis *Rickettsia conorii*[279] and *R. typhi*.[280] The procedure is similar as IFA, but peroxidase is used instead of fluorescein. The main advantage of the immunoperoxidase assay is that one can easily read the results with a light microscope. additionally, immunoperoxidase assay slides can also be kept as a permanent record.

3.10.2.1.9. Western immunoblot assay

Western immunoblot assay is a well established serodiagnostic tool for seroepidemiology and confirmation of serological diagnoses results obtained by conventional tests. The antigen used in this test is gel electrophoresed and electroblotted with sodium dodecyl sulfate. It is mainly useful in differentiating true-positive from false-positive results produced by cross-reacting antibodies. In the Western blot assay, an antibody reaction to individual proteins or the lipopolysaccharide (LPS) of the cell wall can be identified and useful in demonstrating cross-reactions between two biogroups like SFGR and typhus group, and cross reactions between the species within the single biogroup. It was suggested that Western blot can detect antibodies to *Rickettsia spp*. early in the infection than the IFA test can, although the early response is mainly IgM antibodies directed against LPS and the unspecific reactions are likely to be due to LPS

reactions, which are possible to identify with Western blot. Therefore, the benefit of earlier detection by Western blot is lost, because much of the early response is against LPS and hence unspecific.[281,282]

3.10.2.1.10. Line blot assay

Line blot assay is a serological diagnostic method adapted for the diagnosis of MSF and it is found to be almost as specific and sensitive as IFA for the diagnosis of MSF.[281] The line blot immunoassay may be particularly useful for screening many antigens (more than 45 antigens) simultaneously.[283] Thus, line blot assay can be used for patients with atypical or nonspecific clinical presentations for large-scale screening of sera when quantitative titers are not important or when tests against a large number of agents are required.

3.10.2.1.11. Dot blot assay/ Rapid diagnostic test

Dot blot immunoassay is a commercially available rapid diagnostic test, used to screen patients suspected of having scrub typhus infection.[284] This assay tests for the strain antigens such as Karp, Kato, and Gilliam. Scrub typhus rapid diagnostic tests are commercially available from, Standard Diagnostics (SDmICT; Standard Diagnostics, South Korea), Panbio (PBmICT; Panbio, Australia), AccessBio CareStart (ABm/ABt ICT; AccessBio) and Dip s-ticks (Integrated Diagnostics, Baltimore). Both the SDmICT and PBmICT can detect IgM antibodies in the patient's serum. AccessBio has two different test formats that can detect either IgM alone or IgG or total scrub typhus specific antibodies in patient's serum. The earlier assays incorporated the purified antigens of the various prototypes of *O. tsutsugamushi* namely Karp, Kato and Gilliam. The recent advances in the molecular biology had led to the synthesis of recombinant

antigens such as r56-kDa type specific antigens. Newer RDTs have incorporated the newer genotypes in addition to the prototype strains. The RDTs can be as sensitive and specific compared to complex confirmatory assays, however these diagnostic assays must be validated against gold standard test before they are incorporated into routine diagnostic purposes.

3.10.2.2. Direct Diagnosis

3.10.2.2.1. Immunodetection of rickettsia in blood and tissues

Direct detection of rickettsiaal organism in blood or tissues is helpful in confirmation of rickettsial diseases even before seroconversion. The methodology is widely used for the diagnosis of RMSF and MSF and in some cases it was proved to be successful in detection of murine typhus also. [285] The common samples used are skin biopsy with a rash around the lesion, preferably petechial lesions, and tache noire specimens.[87] Samples can be tested fresh or after formalin fixation and paraffin embedment. Recent advances in the immunodetection assay are the development and validation of immunologic detection of *R. conorii* from the circulating endothelial cells which are recovered from whole blood by using immunomagnetic beads coated with an endothelial cell-specific monoclonal antibody.[87] The sensitivity of this method is 50% and does not appear to be influenced by the previous antibiotic therapy or the presence of specific antibodies, as in the case of culture, but it can be used as a prognostic indicator as the level of the CECs increases with the severity of the infection.[223] The utility of the immunodetection technology for scrub typhus diagnosis is yet to be evaluated.

3.10.2.2.2. Isolation of rickettsiae

The rickettsiae can be isolated from several samples such as buffy coat of heparinized blood, defibrinated whole blood, triturated clot, plasma, skin biopsy, necropsy, tissue and directly from arthropod vectors. As most of the rickettsial diseases exhibit indistinguishable clinical manifestations, the isolation of the particular organism from the samples followed by their molecular characterization is most important for the discovery of new rickettsial diseases.

3.10.2.2.3. Animal inoculation

Animal inoculation test are regularly used for research purposes and performed at reference laboratories. During the initial days, embryonated chicken egg yolk sacs were widely used for the growth of rickettsia,[87] but they are now replaced by cell culture systems. Inoculation in to guinea pigs has also been demonstrated.[286] White mice is the choice of animal used for the experimental identification of rickettsial diseases. During inoculation, whole blood from the febrile patient suspected of rickettsial infection would be injected intraperitoneal into white mice. The mouse is observed for the signs of illness or death. Giemsa stained impression smears can be performed on the surface of the spleen or the peritoneum to confirm rickettsiaemia. The isolation rates are less than 50% as the test generally requires weeks to confirm the presence of rickettsia in the blood. Animal inoculation is remains helpful in situations where it is required to isolate the organism from postmortem samples, which are generally contaminated with other bacteria.[87]

3.10.2.2.4. Cell cultures

The ability to isolate a microbial pathogen in culture is definitive in diagnosis of disease and crucial in characterizing the microbes' genotypic and phenotypic features. In fact, being able to culture a disease causing microorganism proves the second and third criteria of Koch's postulates.[287] With the initiation of modern molecular biological techniques in the past couple of decades, the presence of a pathogen's genetic material in a host's tissue sample would be enough for a diagnosis. However, molecular techniques alone are currently unable to provide crucial information accessible only via culture such as pathogen-host cell interactions. Cell culture, demonstrated for more than 60 years, is currently the most widely used method for isolation of rickettsiae from clinical samples. Isolation of *R. rickettsii* from blood has been achieved by using a primary monocyte culture.[87] Later, a tube culture containing an L929 mouse fibroblast cell monolayer was also introduced for the recovery of *R. rickettsii* and *O. tsutsugamushi* from blood samples.[122,289] More recently, the shell vial assay, a commercialized method used for cytomegalovirus culture and early antigen detection, was adapted for the detection of *R. conorii* from extracts of blood, animal and human tissue, with detection of the microorganism being possible in 48 to 72 hours in most of the cases.[87] The small surface area of the cover slip containing cells enhances the ratio of the numbers of rickettsia to the numbers of cells and allows better recovery. Inoculation can be made onto two types of cells. The Vero or L929 cells have shown better and faster recovery of rickettsiae, especially from heavily infected samples, than HEL or MRC5 cells.[290] However, HEL or MRC5 cells also have the advantage that once a monolayer is established, contact inhibition prevents further division of the cells. Cell culture is now one of the most

effective methods available to researchers for isolating *Rickettsia spp*. and other *Rickettsia*-like organisms.[291]

3.10.2.2.5. Polymerase chain reaction

The earliest documented molecular biology-based identification method was based on PCR-restriction fragment length polymorphism (RFLP) analysis of the gene encoding the *OmpA* protein, which allowed the differentiation of the nine SFG rickettsiae studied.[292] In 1993, Furuya *et al.* have described a nested PCR method for the identification of *Orientia* species by targeting 56 *kDa* gene from blood samples.[293] The introduction of PCR, a nucleic acid testing method has revolutionized the detection and diagnosis of rickettsial infections.[294] With access to a laboratory equipped to perform PCRs on patient samples, accurate diagnosis can be obtained within 3 days of onset of the illness, well before the seroconversion in patient. Different PCR techniques have been developed allowing for rapid detection of *Rickettsia and Orientia* sp by targeting various genes. Well described target genes for the identification of Rickettsia sp. are citrate synthase (*glt*A) OmpA (*omp*A) OmpB (*omp*B) 16S rRNA, gene D (*sca4*), and 17*kDa*.[87,248,301] The 47 *kDa*, 56 *kDa* and groel genes are the most targeted specific genes for the identification *Orientia spp*.[293,295,296] The implementation of real-time PCR (qPCR) in rickettsial detection further increased the sensitivity of PCR assays and allows for the quantification of rickettsiae in a sample.[297] Speciation of rickettsiae can now be performed using the criteria proposed by Fournier *et al*.[73] (Figure- 02) and it has been adopted worldwide as the standard for identifying rickettsial species by PCR.

Several clinical samples can be used for the PCR amplification of rickettsial DNA. Skin biopsy specimens and peripheral blood mononuclear cells are regularly used

in specialized laboratories but can be used in any laboratory with PCR facilities.[298,299] CECs concentrated in the buffy coat obtained after decantation of heparinized blood can be used, but the cells must be treated with heparinase prior to PCR amplification. Blood clots, whole blood, or serum has also been successfully used in several studies for the detection of *Rickettsia* and *Orientia* species, [87,239,293] however, *R. conorii* DNA could not be amplified when serum was used.[87] Rickettsial DNA used as a template for PCR amplification can also be extracted from tache noire specimens,[298] cerebrospinal fluid[87] or paraffin-embedded tissues.[300] Detection of rickettsial organism directly from arthropod vectors is also described by many researchers.[301] Same PCR-based detection method can be used for detection of rickettsiae from arthropods.[87]

Unfortunately, the rapid PCR tests or definitive serologic methods are still unavailable in many parts of Southeast Asia where rickettsial infections are rampant, and where the local population would be benefited from those tests. Thus, presumptive clinical diagnosis by trial of doxycycline remains the standard practice. [302]

3.10.2.3. Differential diagnosis

The diagnostic difficulty in rickettsiaosis becomes more complicated due to the non specific signs and symptoms and co-existence of other tropical febrile illness. The differential diagnosis would include typhoid fever, dengue, malaria, measles, rubella, leptospirosis, secondary syphilis, disseminated gonnococcal infection, respiratory tract infection, gastroenteritis, meningococcemia, immune complex vasculitis, idiopathic thrombocytopenic purpura, thrombotic thrombocytopenic purpura, infectious mononucleosis and adverse drug reaction. Invasive meningococcal disease may not be reliably distiguished from rickettsial disease clinically; hence one may need to treat for

both conditions, after sending cerebrospinal fluid and blood for appropriate studies. In India, the common differential diagnosis for AFI would be rickettsiosis, typhoid, dengue, malaria and leptospirosis. Presently, no assay is available to identify all the tropical febrile illness in a single test; however, there are continuous attempts to devise an assay which can simultaneously detect several tropical diseases.[250]

3.11. Treatment

Doxycycline (100 mg, twice a day) is recommended for treatment of murine typhus and all SFGR infections in adults and children (weighing >45 kg), and should be continued for a minimum of 5 to 7 days in total or three days after the resolution of symptoms.[129] For children weighing less than 45 kg, doxycycline at a dose of 0.9 mg/kg/ day in two divided doses is recommended. Chloramphenicol (250–500 mg every 6 hours) can be used to treat women in first or second trimester of pregnancy, and quinolones may be used as an alternative.[129] However, murine typhus may show variable responses to quinolones, and treatment failure has been reported with ciprofloxacin.[57]

Doxycycline (100 mg, twice a day) or chloramphenicol (250–500 mg every 6 hours) can be used to treat scrub typhus. The *O. tsutsugamushi* strain Kato has been reported to be naturally resistant to quinolones.[57] Reduced susceptibility to doxycycline and chloramphenicol has also been reported from Chiangrai in northern Thailand.[57] A randomized controlled study from the same area showed that treatment with rifampicin (300–450 mg, twice a day) for one week was superior to doxycycline in terms of time to defervescence and relapse rates. However, combination therapy with rifampicin and doxycycline did not show any added efficacy. [303] A prospective, open-label, randomized

trial in areas where reduced susceptibility to tetracyclines is present showed that, single dose of azithromycin (500 mg) may be equally effective as a seven-day course of doxycycline (100% versus 93.5% cure; P = 0.12) with similar median time to defervescence (21 hours versus 29 hours; P = 0.97).[57]

Azithromycin is also considered safe in pregnant women. A five-day regimen of 800 mg/day of telithromycin may have efficacy similar to that of doxycycline in treatment of mild-to-moderate scrub typhus.[57] Optimal duration of treatment with doxycycline is uncertain, and courses as short as three days have been studied.[57]

Doxycycline is considered safe in children < 9 years of age, has limited risk of dental staining with short courses, and fever resolution is seen within 48 hours in most.[136]

3.12. Prevention

Personal protection against ticks (wearing proper clothing and use of repellants) and other arthropods remains an important measure to get protected against rickettsial infections. In case of exposure or bites, quick removal of ticks proves extremely beneficial in prevention of infection. Attempting to control the tick reservoir is not a realistic approach, and use of antibiotics following tick exposure is not currently indicated to prevent rickettsial infection.[2]

3.12.1. SFGR

A recently improved killed chicken embryo vaccine has shown that it may provide partial protection against RMSF and ameliorate the illness when it occurs. Avoidance of contact with and control of house mouse infestations is important to prevent acquisition

of rickettsial pox infection. For Boutonneuse fever, natural immunity develops following infection. Effective vaccines are still not available.[2,57]

3.12.2. Louse-borne (epidemic) typhus

Prompt removal of louse from individuals and use of insecticides to treat clothing if they are found are effective preventive measures against the spread of louse-borne typhus. Killed vaccines were shown to reduce mortality rates but were not effective in prevention of disease. Brill-Zinsser disease is recrudescence of primary louse-borne epidemic typhus, may not produce severe illness as the immunity developed during epidemic typhus infection.

3.12.3. Murine Typhus

Prevention is primarily by controlling the flea and rat populations. Insecticides should be used before rodenticides to prevent rat fleas from seeking alternate hosts if rats are no longer available.[2,57]

3.12.4. Scrub Typhus

The disease is best prevented by the use of personal protective measures including repellents. People entering an exposed area wear closed in footwear such as

boots with socks, and long trousers. Exposed areas of skin and clothing itself should be treated with mite repellents. Those people working in infested areas should consider impregnating clothing with permethrin. Prophylactic treatment usually consist oral chloramphenicol or tetracycline given once every 5 days for thirty-five days or weekly doses of doxycycline during and for 6 weeks after exposure have both been shown to be effective regimes. No effective vaccine has been developed for scrub typhus.[2,57]

MATERIALS & METHODS

CHAPTER -04

MATERIALS & METHODS

4.1. Study design and Study duration: This work is a cross sectional study conducted from the year 2012 to 2016.

4.2. Study population:

Patients of all age-groups and both sexes attending OPD and admitted in inpatient departments of Shri B.M. Patil Medical College Hospital and Research centre Vijayapura, District hospital, Vijayapura and peripheral centres (in and around Vijayapura) presenting with consistent fever, headache, myalgia, rashes.

4.3. Ethical committee clearance: Institutional ethical clearance obtained from BLDEUs Shri B.M Patil medical college, Vijayapura to carry out the study.

4.4. Sample Size: 572 clinically suspected patients.

Considering average proportion of Rickettsial infection reported during the year 2009 - 2011 (60%), with 4% marginal error and 95% confidence interval, the sample size was calculated using the formula;

$$n = (Z)^2 \times p \times (1-p) \over l^2$$

n = 572

p = Average proportion – 60%

l = Marginal error – 4%

4.4. Inclusion Criteria:

Patients of all age groups irrespective of sex, attending OPD and hospitalized with fever without a source; presence of one or more of the following clinical features: head ache, myalgia, rash, edema, eschar, and tick bite or tick exposure.

4.5. Exclusion Criteria:

Cases already diagnosed as other febrile diseases at the time of sample collection.

4.6. Sample collection

4.6.1. Collection of Blood:

After obtaining the informed consent, 5 ml of venous blood was collected from each patient in a plain tube under aseptic precautions.

4.6.2. Sample Processing:

4.6.2.1. Serum separation

Tubes were kept undisturbed at room temperature for 30 minutes to allow the blood to clot. After clotting, tubes were centrifuged at 1000xg for 15 minutes to separate the serum. 1 to 1.5ml of serum was separated from each tube and transferred to sterile 2ml screw cap vials labeled with identification number.

4.6.2.2. Storage of samples

Both the serum samples and blood clots were preserved at -20°C for further analysis.

4.7. Tests performed for serodiagnosis of Rickettsial infection

4.7.1. Weil felix tube agglutination test:

The Weil – felix reaction is an agglutination test in which sera are tested for agglutinins to the 'O' antigens of certain non-motile *Proteus* strains i.e. *Proteus vulgaris* OX-19, *Proteus vulgaris* OX-2 and *Proteus mirabilis* OX-K.

Test Procedure:

- The test tubes were arranged in three rows each row containing 8 tubes.
- Added 1.9 ml of physiological saline to tube No.1.
- To each of the remaining tubes (2 to 8) added 1 ml of physiological saline.
- Added 0.1 ml of serum sample to the first tube in each row and mixed well.
- Serially diluted by mixing and transferring 1 ml diluted serum from first tube into second and up to 7^{th} tube. 1 ml from the seventh tube in all rows was discarded.
- Sample dilution achieved after the serial dilution from tube No. 1 to 7 in each set was 1:20, 1:40, 1:80, 1:160, 1:320, 1: 640 and 1:1280 respectively. Tube No. 8 in all the set was negative control.
- To all the tubes (1 to 8) of each set added one drop of the respective well mixed PROGEN antigen suspensions and mixed well.
- Covered all the sets and Incubated at 37°C (approximately 18 hours).
- After the incubation tubes were observed for the agglutination by dislodging the sediment gently.
- Interpreted and results were recorded in the worksheet and in predesigned proforma created in MS Excel 2007.

4.7.2. IgM ELISA test for the serological confirmation of SFGR (*R. conorii*):

Rickettsia Conorii IgG/IgM ELISA (Vercell Microbiologists, GRANADA SPAIN) was used to detect the presence of IgM antibodies in patient's serum. In this method, corresponding IgM antibodies present in the serum react with the R. conorii antigen, strain Moroccan (ATCC VR-141) coated on ELISA wells.

Test Procedure:

- Brought all the reagents to room temperature and mixed properly by shaking before use.
- Placed required number of wells the plate and added 25 µl of VIRCELL IgG sorbent to each wells, except controls wells.
- Added 5 µl of sample and 75 µl of the serum diluent to respective wells, and mixed well using same tip.
- Dispensed 5 µl of the positive control, 5 µl of the cut off Control (in duplicate) and 5 µl of the negative control to the corresponding wells containing100 µl of the serum diluent and mixed well.
- Plate was covered with a sealing sheet and incubated at 37±1ºC for 45 min.
- Removed the seal, aspirated liquid from all wells and washed five times with 0.3 ml of washing solution per well, drained off any remaining liquid.
- Added 100 µl of IgM conjugate solution into each well immediately.
- Covered with a sealing sheet and incubated in incubator at 37±1ºC for 30 min.
- Aspirated liquid from all wells and washed five times with 0.3 ml of washing solution per well per cycle, drained off any remaining liquid.
- Immediately added 100 µl of substrate solution into each well.

- Incubated at room temperature for 20 minutes protected from light.
- Added 50 µl of stopping solution into all wells immediately.
- Absorbance was red with ELISA plate reader at 450/620 nm and recorded results.
- Antibody index was calculated by using the below formula for interpreting the results.

Antibody index = (sample O.D / cut off serum mean O.D) x 10

- Results were interpreted as positive, negative and equivocal on the basis of antibody index values.
- Samples with antibody index >11 considered as positive, samples with antibody index <9 were considered as negative and samples with antibody index between 9 -11 were considered as equivocal and repeated those samples in next run.

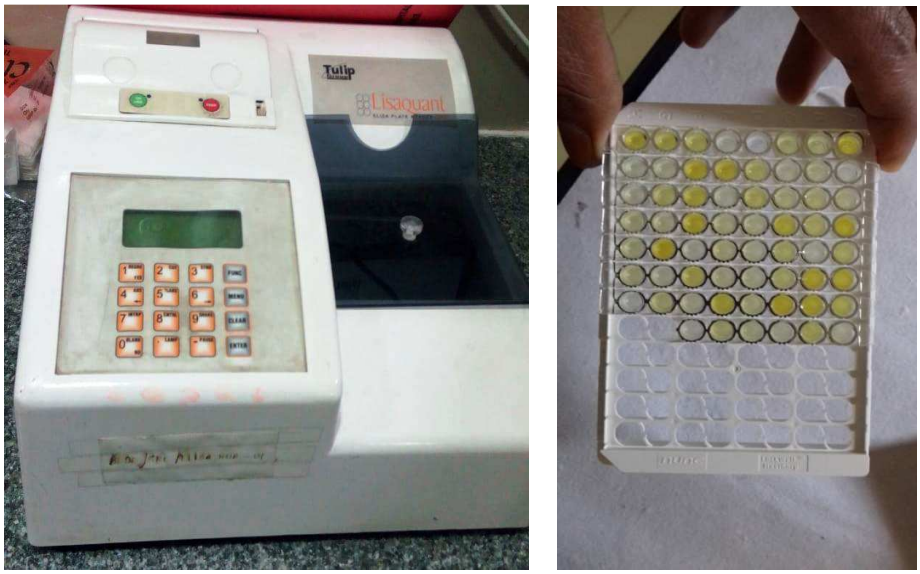
4.7.3. IgM ELISA test for the serological confirmation of Scrub typhus infection:

Scrub Typhus Detect™ IgM ELISA kit (InBios International Inc, USA) was used to detect the presence of IgM antibodies in patient's serum. Recombinant 56 *kDa* type specific antigen of *O. tsutsugamushi*, Karp, Kato and Gilliam genotypes were coated in the ELISA wells. The IgM ELISA test was initially standardized using serum samples from healthy blood donors and the OD cutoff of 0.5 was determined by taking the mean OD plus three times of the Standard Deviation.

Test Procedure:

- Brought all the reagents to room temperature and mixed properly by shaking before use and arranged required numbers of wells in the plate.
- Patient's sera were diluted to 1/100 proportion by mixing 4µl of individual test sera with 396µl of Sample dilution Buffer.

- Dispensed 100 µl of the diluted test sera and controls to respective wells.
- Covered the plate with plate covers, incubated the plate at 37°C for 30 minutes in a water bath.
- Wells were washed six times with the 300 µl of 1X Wash Buffer per well in each wash cycle.
- Added 100 µl of Ready to Use Enzyme-HRP Conjugate for Scrub Typhus IgM into all the wells and incubated as before.
- Washed the wells six times with the 300 µl of 1X Wash Buffer per well in each wash cycle.
- Added 150 µl of EnWash into all wells and incubated at room temperature for 5 min without any cover on plate.
- Wells were washed six times with the 300 µl of 1X Wash Buffer per well in each wash cycle.
- Added 100 µl of Liquid TMB substrate into all the wells, incubated the plate at room temperature for 10 minutes in a dark place without any cover on the plate.
- After the incubation, added 50 µl of Stop Solution into all wells and incubated at room temperature for 1 minute without any cover on the plate.
- Absorbance was red with ELISA plate reader at 450 nm and recorded results.
- The cut off OD value fixed for interpreting the result was 0.5, which was determined experimentally following kit protocol.



A **B**

Fig-15: ELISA Reader (A), ELISA plate ready to read (B).

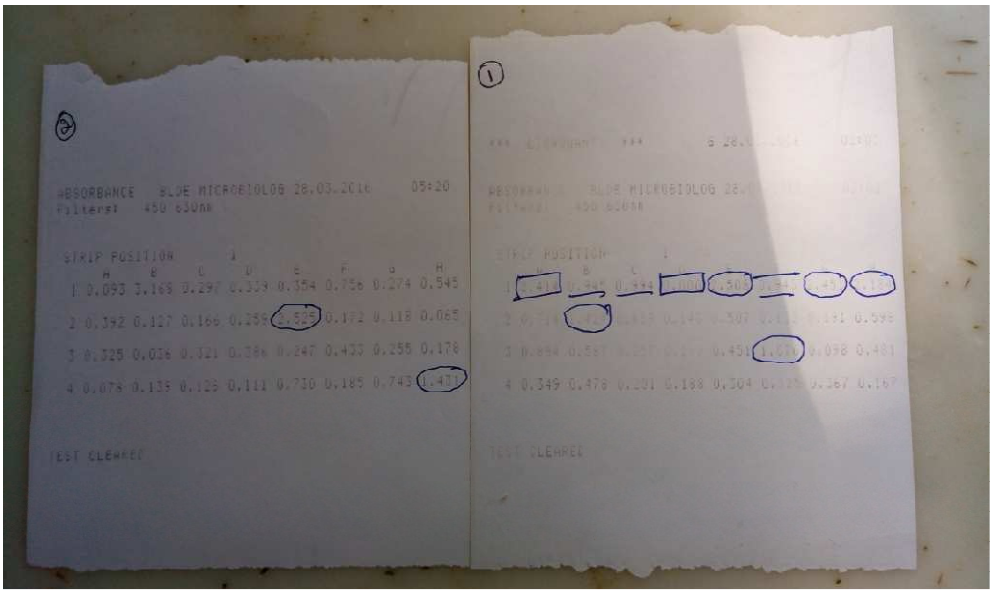


Fig-16: ELISA Report print.

4.8. Molecular methods - Polymerase Chain Reaction

For the molecular confirmation of the cases, Nested PCR was performed according to the methods previously described by Furuya *et al.*[293] for the detection of *Orientia spp*. and Roux V *et al.* for the detection of SFGR.[306] Only ELISA positive cases were included in the molecular detection. Entire Molecular work was carried out at Molecular Biology laboratory, NITM, ICMR Belgaum with the permission of then Director In Charge – Dr. S.L. Hoti.

4.8.1. DNA Extraction using QIAmp blood mini kit (Qiagen, USA)

- Blood clot was homogenized by heating gently in a water bath and vortexing intermittently.
- Added 20µl *Proteinase K* into the bottom of a 1.5 ml microcentrifuge tube
- Added 200µl of homogenized blood to the tube and mixed by slow vortexing.
- Added 200µl Buffer AL to the sample and mixed by pulse-vortexing for 15 seconds, incubated at 56°C for 10 min in a water bath.
- Briefly centrifuged the tube to remove drops from the inside of the lid
- Added 200µl of ethanol (96–100%) to the sample, and mixed again by pulse-vortexing for 15 sec. After mixing, briefly centrifugeed the tube to remove drops from the inside of the lid.
- Carefully transferred the content to QIAamp Mini spin column placed in a 2 ml collection tube. Closed the cap, and centrifuged at 8000 rpm for 1 min.
- Placed the QIAamp Mini spin column in a clean 2 ml collection tube and added 500µl Buffer AW1, closed the cap and centrifuged at 8000 rpm for 1 min.

- Placed the Mini spin column in a new 2 ml collection tube, added 500µl Buffer AW2 and centrifuged at full speed (14,000 rpm) for 3 min.
- Placed the Mini spin column in a new 2 ml collection tube, centrifuged at full speed for 1 min to remove buffer completely.
- Placed the Mini spin column in a clean 1.5 ml microcentrifuge and added 200µl Buffer AE, incubated at room temperature for 1 min, and centrifuged at 8000 rpm for 1 min.
- Labeled the tubes containing Eluted DNA.
- DNA was electrophoresed in 1% agarose gel and visualized in Gel documentation system (Syngene, USA)

4.8.2. Primers used for PCR in the study:

Primer sequences were chosen from the published articles. [248,293] All the Primers used in the present study were procured from IDT through Sai Scientific company, Chennai.

In the nested PCR for the detection rickettsiae specific gltA gene, primers used in first round of amplification were RpCS.877p & RpCS.1258n, and in second round primers used were RpCS.780p & RpCS.1258n.[248]

In the nested PCR for the detection of SFGR specific ompA gene, primers used in first round of amplification were Rr190k.71p & Rr190k.720n, and in second round primers used were Rr190 k.71p & Rr190k.602n.[248]

In the nested PCR for scrub typhus, primers used in first step were 34 and 55 and primers used in second step were 10 and 11 as described by Furuya *et al.*[293]

Table-02: IDT Primers used for amplification of Spotted Fever Group Rickettsiae and Scrub typhus by nested PCR method.

Sl. No	Gene targeted	Primer Name	Primer Sequence 5'- 3'	Amplicon Size (bp)	Reference
1	gltA For genus Rickettsia	RpCS.877p	GGGGGCCTGCTCACGGCGG	338 bp	JAJ Prakash *et al* [248]
		RpCS.1258n	ATTGCAAAAAGTACAGTGAAC		
		RpCS.896p	GGCTAATGAAGCAGTGATAA	382 bp	
		RpCS.1233n	GCGACGGTATACCCATAGC		
2	OmpA For SFGR	Rr190k.71p	TGGCCAATATTTCTCCAAAA	650 bp	
		Rr190k.720n	TGCATTTGTATTACCTATTGT		
		Rr190 k.71p	TGGCGAATATTTCTCCAAAA	532 bp	
		Rr190k.602n	AGTGCAGCATTCGCTCCCCCT		
3	56kDa For Scrub typhus	F- P34	TCA AGC TTA TTG CTA GTG CAA TGT CTGC		Furuya *et.al* from Japan [293]
		R- P55	AGG GAT CCC TGC TGC TGT GCT TGC TGCG		
		F- P10	GAT CAA GCT TCC TCA GCC TAC TAT AAT GCC	483 bp	
		R- P11	CTA GGG ATC CCG ACA GAT GCA CTA TTA GGC		

4.8.4. Amplification:

Reaction mixture was prepared in 200 µl PCR tubes and amplification of the DNA was performed in the Thermal Cycler (ESCO). The steps involved in amplification were initial denaturation, 35 cycles of denaturation, annealing and elongation or extension, and final extension. The nested PCR constituted two rounds of amplification

steps. In the first round, 5µl of the original DNA was used as template. In the second round, 5 µl of the amplification product of first round was used as template.

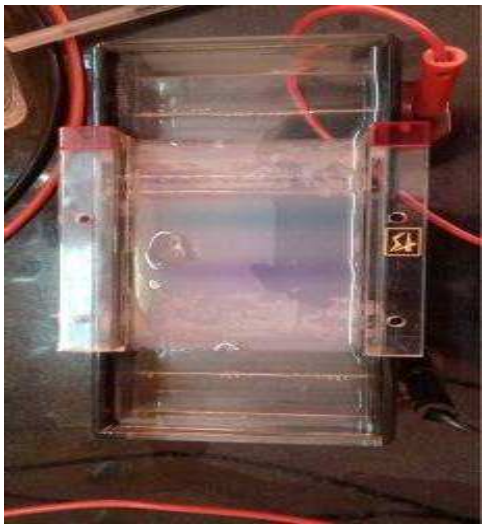


Fig-17: Thermal Cycler and Gel electrophoresis unit

Table-03: PCR reaction mixture

Sl. No	Reagents	Volume
1	Promega green Master mix	25µl
2	Primer 1	2 µl
3	Primer 2	2 µl
4	DNA template	5 µl
5	Water	16 µl
	Total Volume of reaction mixture	50 µl

Known positive DNA for 56-kDa TSA of *O. tsutsugamushi* and distilled water was used as positive and negative control in the n- PCR for scrub typhus respectively.

As the *Rickettsia* or SFGR positive sample was not available, made attempts to standardize the PCR for the identification of *Rickettsia conorii* by applying the same PCR conditions described in published articles.[248]

Table-04: Nested PCR Amplification conditions for different primers used in the study

Gene	**Initial Denaturation**	**Denaturation**	**Annealing**	**Extension**	**Final extension**	**No of cycles**
gltA	95°C for 10 min	95°C for 30sec	44°C for 30sec	65°C for 2 min	65°C for 10 min	35
OmpA	95°C for 10 min	95°C for 30sec	55°C for 30sec	65°C for 2 min	65°C for 10 min	35
56kDa	95°C for 10 min	95°C for 30 sec	55°C for 1 min	70°C for 1 min	70°C for 10 min	35

4.8.5. Gel documentation

Once the PCR cycle completed, the amplicons were loaded onto a 1 % agarose gel (Sigma Aldrich) containing 0.5 mg/ml of ethidium bromide. A 100bp DNA ladder was also added to a well to allow post run band size determination. The gel was immersed in 1x TAE buffer and a voltage was applied across it using a Power Pac 300 (Bio-Rad, USA) power supply to separate the different size DNA bands. The gel was visualized in Gel documentation system system (Syngene, USA). (Vilber Lourmat, Labtech international, East Sussex). The sample was considered positive for scrub typhus, if there was an amplified product of size approximately 483-bp (Figure 8.4).

4.8.6. DNA Sequencing and Phylogenetic analysis

The 483-bp fragment of 56-kDa TSA gene was amplified using overlapping primers 10 and 11, and the amplicons were sent to Eurofins genomics India Pvt. Ltd, Bangalore for further purification and sequencing. PCR products were purified using a QIAquick gel extraction kit (Qiagen, USA) and sequenced bidirectionally using Applied Biosystems model 3730xl/3730XLPA-19137-17.

Quality of sequences was checked with Finch TV Version 1.4 and consensus sequence was generated using DNA Dragon-DNA Sequence Contig Assembler Software V1.5.61. Further consensus sequences were queried against NCBI BLAST programme to confirm the successful PCR amplification and identify the sequenced sample. Sequences were aligned with reference sequences of *Orientia tsutsugamushi* in MEGA 7 software. Phylogenetic trees were constructed using already uploaded genotype sequences data available in the NCBI website and sequence data obtained for the cases in the present study. The tree displayed the percentage of phylogenetic divergence of each sequence, represented numerically by bootstrap values displayed at each joint on the basis of the branch length, and the stability of each branch.

4.9. Statistical Analysis

The clinical and laboratory data were entered into MS excel. Categorical data expressed as percentages and continuous data summarized using mean and standard deviation. Performance characteristics of serological assays have been calculated using MedCalc easy to use statistical software for windows. Test of association was performed using Pearson's Chi square test, and a p-value of <0.05 was considered statistically

significant. Statistical software package SPSS version 22 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) was used to analyse the data.

RESULTS

CHAPTER 05

RESULTS

5.1. Demographic details of suspected cases included in the study:

A total of 572 blood samples were collected from patients who were clinically suspected of having rickettsial infection during the period 2013 to 2017. study as per the inclusion c criteria. Among 572 cases, more number of suspect cases observed in the age groups 16-30 and 46 and above in both males and females. Among sexes, proportion of suspect cases was comparatively high in males with 63.46% share.

Table-05 . Age and gender wise distribution of suspected cases

Age in years	Male (%)	Female (%)	Total (%)
0 to 15	64 (11.18)	42 (7.34)	106 (18.53)
16 to 30	115 (20.10)	72 (12.58)	187 (32.69)
31 to 45	71 (12.41)	37 (6.46)	108 (18.88)
46 & above	113 (19.75)	58 (10.13)	171 (29.89)
Total	**363 (63.46)**	**209 (36.53)**	**572 (100)**

(n=572) Chi-square value = 1.4943 at D.F = 3 and p = 0.683 > 0.05

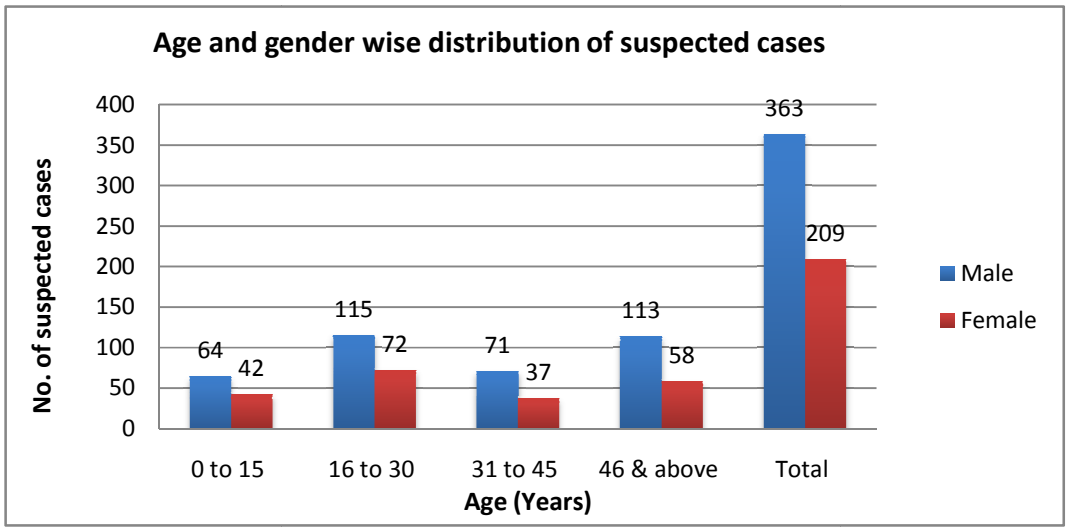


Fig-18 . Graphical representation of Age and Gender wise distribution of suspect cases

5.2. Serodiagnosis by Weil felix test- Titre wise results for the cases subjected in the study.

Serial dilution was made up to 1:1280. Highest titre observed in the study was 1:640 with OX2 antigen. Agglutination observed in ≥1:160 dilutions with any one of the antigen was considered as positives. Total WF positive cases were 213. Maximum seropositivity was observed with the antigen OX2.

Table 06: Titre wise Weil felix test results for cases subjected in the study (n=572).

Titre	Weil felix antigens		
	OX2	OX19	OXK
1:1280	0	0	0
1:640	01	0	0
1:320	32	0	18
1:160	147	44	45
1:80	57	13	156
1:40	97	79	94
1:20	75	130	64
No agglutination	163	306	195
Total	572	572	572

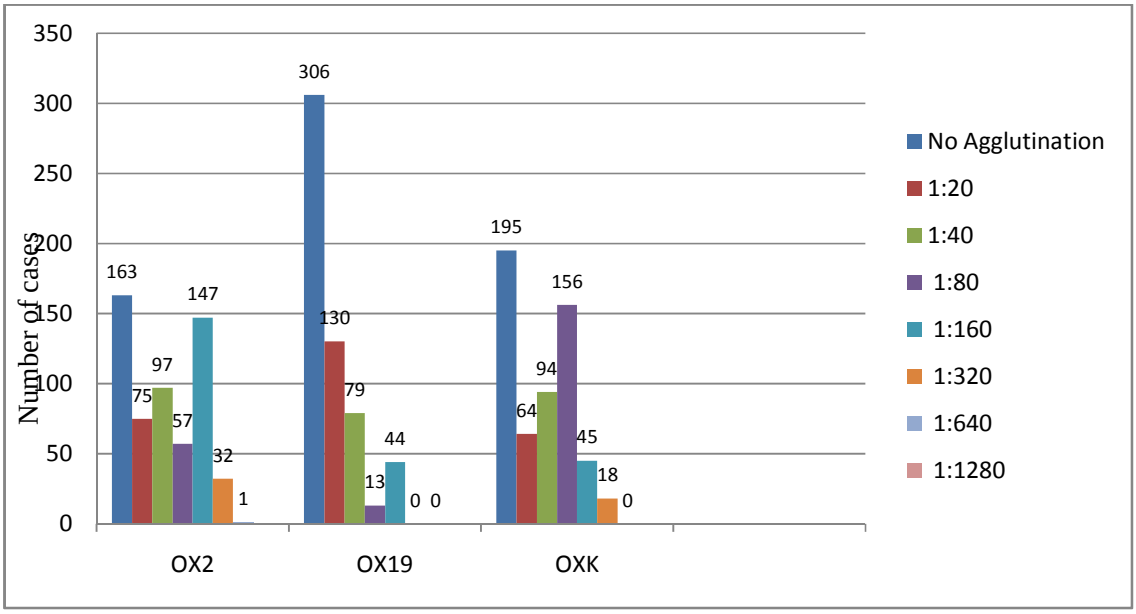


Fig- 19: Graphical representation of Weil felix test results for the cases included in the study

5.3. Age and Gender wise distribution of Seropositivity by Weil felix test

There is no statistically significant difference between Male and Female in seropositivity by Weil Felix Test.

Table-07. Age and Gender wise distribution of seropositivity by Weil felix (n=213)

Age in years	Male (%)	Female (%)	Total (%)
0 to 15	16 (7.51)	17 (7.98)	33 (15.49)
16 to 30	32 (15.02)	30 (14.08)	62 (29.10)
31 to 45	31 (14.55)	19 (8.92)	50 (23.47)
46 & above	43 (20.18)	25 (11.73)	68 (31.92)
Total	122 (57.27)	91 (42.72)	213 (100)

Chi-square value = 3.30 at D.F = 3 and p = 0.348 > 0.05

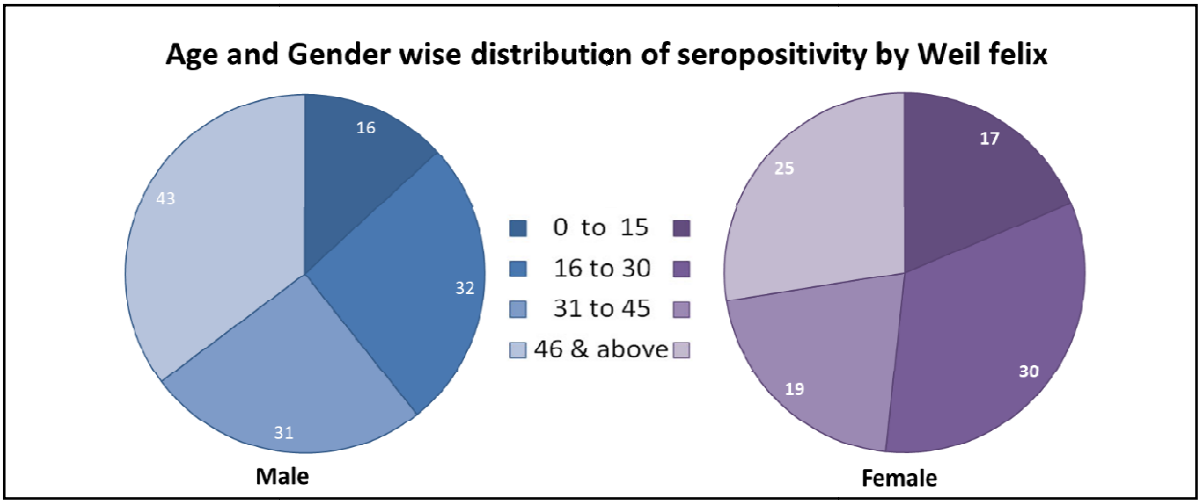


Fig -20. Graphical presentation Age and Gender wise distribution of seropositivity by Weil felix

5.4. Year wise data of cases tested and seropositivity by Weil Felix test

Highest number of clinically suspect cases were observed during the year 2014 with moderate positivity rate by WF test. Significantly high positivity was observed during the year 2016.

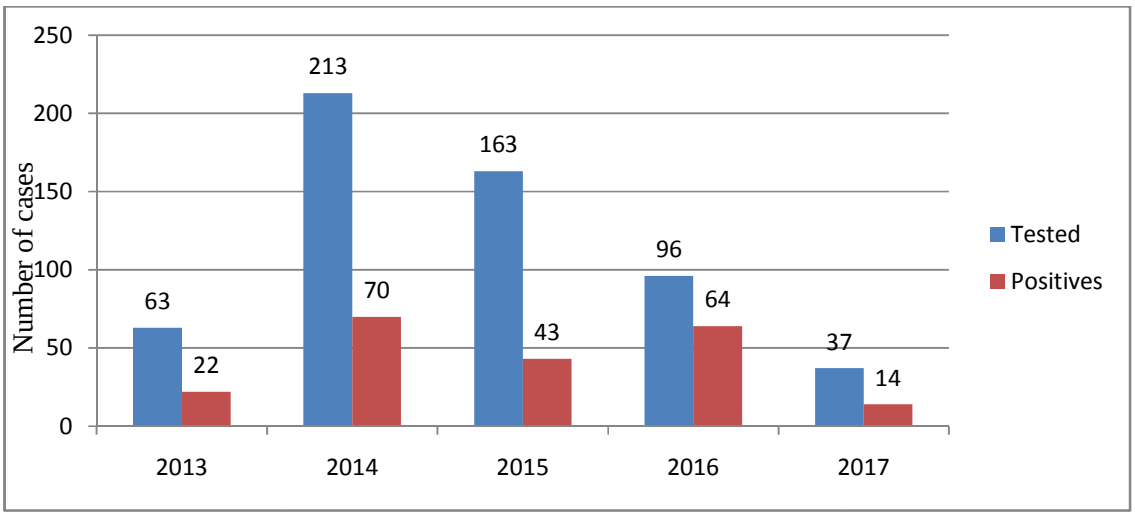


Fig -21. Graphical representation of year wise data of total tested and WF positive cases.

5.5. Month wise distribution of cases screened every year with Weil felix positives.

Significantly more number of cases tested and seropositivity was observed during the cooler months i.e. August to January every year.

Table- 08: Month wise distribution of cases every year with Weil felix positives

Year	2013		2014		2015		2016		2017	
Month	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive
Jan	**15**	**7**	**14**	**3**	**27**	**11**	**9**	**2**	**25**	**12**
Feb	6	2	7	4	9	3	10	7	12	2
March	7	2	10	3	12	1	6	4		
Apr	4	1	4	1	13	2	7	5		
May	5	1	14	3	9	0	6	3		
June	4	0	11	4	15	1	4	3		
July	2	0	25	7	18	5	3	2		
Aug	**5**	**1**	**26**	**7**	**16**	**7**	**9**	**5**		
Sept	**6**	**3**	**31**	**9**	**14**	**5**	**9**	**7**		
Oct	**5**	**2**	**34**	**14**	**11**	**4**	**9**	**7**		
Nov	**2**	**2**	**20**	**8**	**10**	**2**	**10**	**8**		
Dec	**2**	**1**	**17**	**7**	**9**	**2**	**14**	**11**		
Total	63	22	213	70	163	43	96	64	37	14

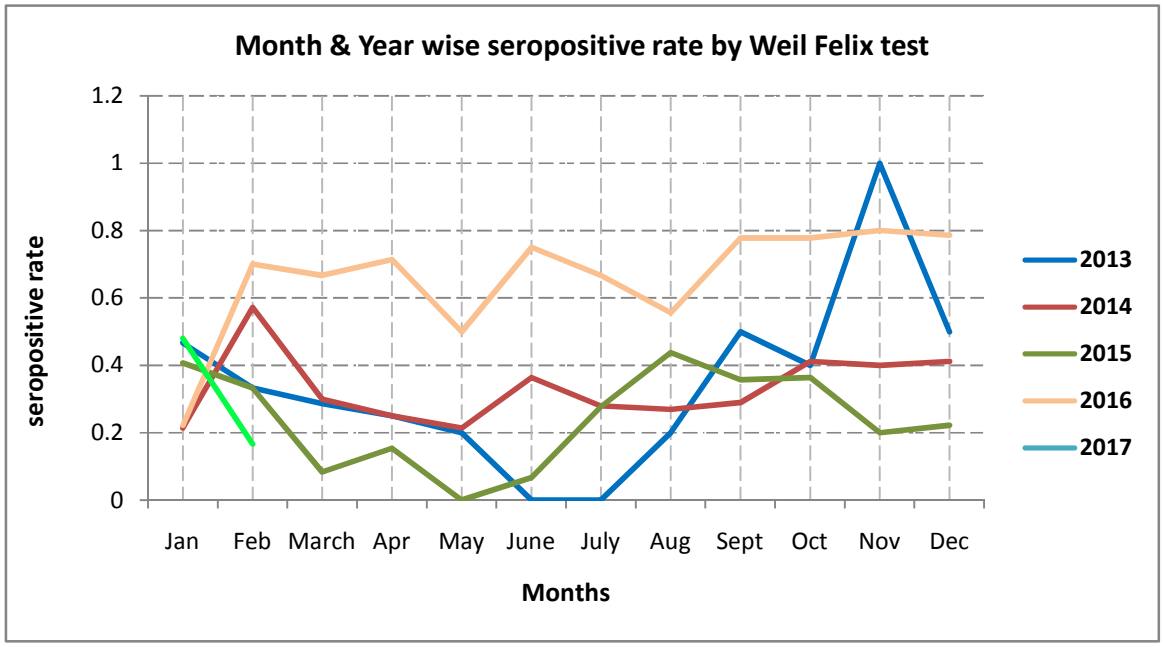


Fig-22: Graphical presentation of Month & Year wise seropositivity rate by Weil F test.

5.6. Year wise data of seropositivity rate by both Weil felix and IgM ELISA

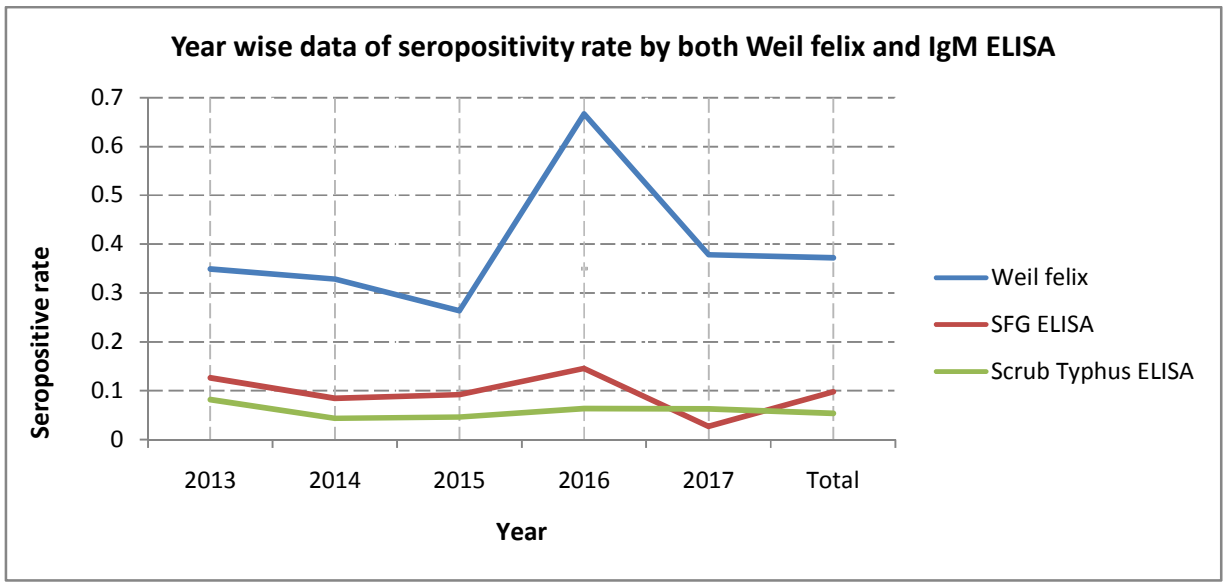


Fig-23. Year wise data of seropositivity rate by both Weil felix and IgM ELISA

Table-09: Year wise data of serodiagnosis by both Weil felix and IgM ELISA for *R. conorii* and Scrub typhus

Tests	2013		2014		2015		2016		2017		Total	
	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive
Weil felix (n=572)	63 (11.01)*	22 (34.92)**	213 (37.23)	70 (32.86)	163 (28.49)	43 (26.28)	96 (16.78)	64 (66.6)	37 (6.46)	14 (37.83)	572 (100)	213 (37.23)
Seropositivity rate	0.349206		0.328638		0.263804		0.666667		0.378378		0.372378	
SFG ELISA (n=572)	63 (11.01)*	8 (12.69)**	213 (37.23)	18 (8.45)	163 (28.49)	15 (9.20)	96 (16.78)	14 (14.58)	37 (6.46)	1 (2.70)	572 (100)	56 (9.79)
Seropositivity rate	0.126984		0.084507		0.092025		0.145833		0.027027		0.097902	
Scrub Typhus ELISA (n=432)	49 (11.34)*	4 (8.16)**	162 (37.5)	7 (4.32)	110 (25.46)	5 (4.54)	79 (18.28)	5 (6.32)	32 (7.40)	2 (6.25)	432 (100)	23 (5.32)
Seropositivity rate	0.081633		0.04321		0.045455		0.063291		0.0625		0.053241	

*Out of total tested during study period, ** Out of samples tested during the year

5.7. Age and gender wise distribution of seropositivity for *R. Conorii* by IgM ELISA

In SFG IgM ELISA, though more positivity was observed in the age groups 16-30 and 46 & above, positivity for Male and Female was statistically not significant.

Table-10. Age and gender wise distribution of seropositivity for *R. Conorii* by IgM ELISA

Age in years	Male (%)	Female (%)	Total (%)
0 to 15	07 (12.5)	04 (7.27)	11 (20)
16 to 30	12 (21.42)	07 (12.5)	18 (32.72)
31 to 45	04 (7.14)	02 (3.57)	06 (10.90)
46 & above	11 (19.64)	09 (16.07)	20 (36.36)
Total	34 (61.71)	22 (39.28)	56 (100)

Chi-square value = 0.450 at D.F = 3 and p = 0.93 > 0.05

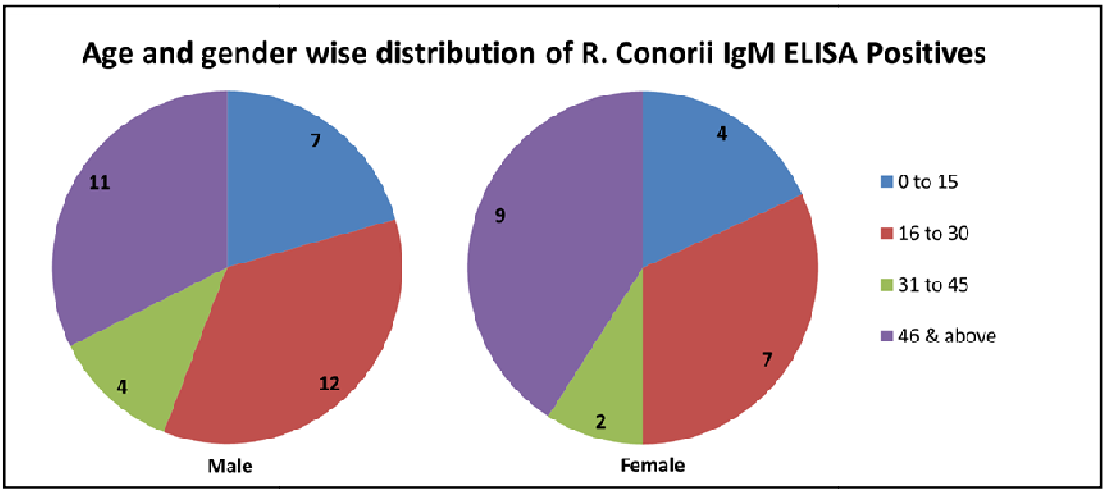


Fig-24. Graphical presentation of age and gender wise distribution of seropositivity by *R. conorii* IgM ELISA.

5.8. Age and gender wise distribution of seropositivity for Scrub typhus by IgM ELISA

Seropositivity for scrub typhus by IgM ELISA in Male and Female was statistically not significant.

Table-11. Age and gender wise distribution of seropositivity for Scrub typhus by IgM ELISA.

Age in years	Male (%)	Female (%)	Total (%)
0 to 15	02 (8.69)	03 (13.04)	05 (21.73)
16 to 30	03 (13.04)	04 (17.39)	07 (30.43)
31 to 45	05 (21.73)	02 (8.69)	07 (30.43)
46 & above	03 (13.04)	01 (4.34)	04 (17.39)
Total	13 (56.52)	10 (43.47)	23 (100)

Chi-square value = 2.28 at D.F = 3 and p = 0.517 > 0.05

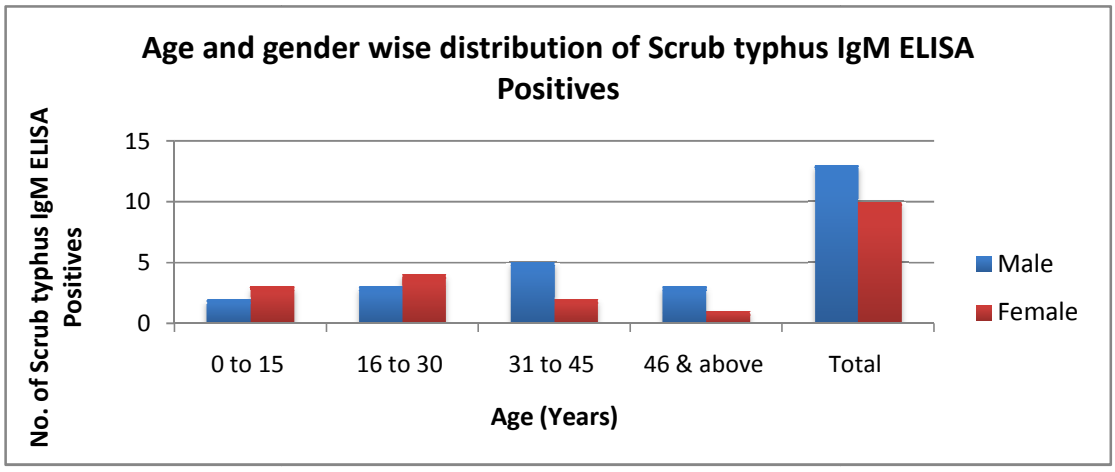


Fig-25. Graphical presentation of Age and Gender wise distribution of seropositivity by Scrub typhus IgM ELISA.

5.9. Gender wise comparison of seropositivity Rate by Weil felix test (OX2) and R. conorii specific IgM ELISA

Statistically highly significant difference was observed. Samples which have shown reaction with only OX2 have demonstrated comparatively high specificity in comparison with ELISA.

Table-12. Gender wise comparison of seropositivity Rate by Weil felix test (OX2) and SFG specific IgM ELISA.

Test	Sample tested			Sample positive			Seropositivity rate		
	Male	Female	Total	Male	Female	Total	Male	Female	Total
Weil felix	363	209	572	110	70	180	30.30%	33.49%	31.46%
IgM ELISA	363	209	572	34	22	56	9.36%	10.52%	9.79%

Chi-square value = 82.1 at D.F = 1 and $p = 0.000 < 0.05$

5.10. Gender wise comparison of seropositivity Rate by Weil felix test (OXK) and Scrub typhus specific IgM ELISA

Statistically highly significant difference was observed. Samples which have shown reaction with only OXK have demonstrated high specificity when compare to ELISA.

Table-13. Gender wise comparison of seropositivity Rate by Weil felix test (OXK) and Scrub typhus specific IgM ELISA.

Test	Sample tested			Sample positive			Seropositivity rate		
	Male	Female	Total	Male	Female	Total	Male	Female	Total
Weil felix	363	209	572	32	31	63	8.81%	14.83%	11.01%
IgM ELISA	279	153	432	13	10	23	4.65%	6.53%	5.32%

Chi-square value = 20.1 at D.F = 1 and p = 0.00 < 0.05

5.11. Performance characteristics of IgM ELISA and Weil–Felix test in detection of SFGR specific antibodies in serum samples.

Table 14. Comparison of Weil felix and IgM ELISA for detection of SFG Rickettsiae. (n=572)

Tests	ELISA Positives	ELISA Negatives	Total	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Accuracy (95% CI)
Weil felix positives	45	135	180	76.2%	69.3%	25%	92.1%	70.48%
Weil felix negatives	11	381	392					
Total	56	516	572					

5.12. Performance characteristics of IgM ELISA and Weil–Felix test with titre > 1:320 in detection of SFGR specific antibodies in serum samples.

Increase in the Sensitivity, specificity and PPV was observed with the increase in the cut off titre i.e. 1:320 and above. Samples with titre of 1:160 were excluded here.

Table 15: Performance characteristics of IgM ELISA and Weil–Felix test with titre >1:320 in diagnosis of SFGR (n=425)

Tests	ELISA Positives	ELISA Negative	Total	Sensitivity	Specificity	PPV	NPV	Accuracy
Weil felix positives	29	04	33	83.5%	92.15%	68.9%	94%	93.1%
Weil felix negatives	11	381	392					
Total	40	385	425					

5.13. Performance characteristics of IgM ELISA and Weil–Felix test in diagnosis of Scrub typhus specific antibodies in serum samples

Table-16: Performance characteristics of IgM ELISA and Weil–Felix in diagnosis of Scrub typhus**(n=432)**

Tests	ELISA Positives	ELISA Negatives	Total	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Accuracy (95% CI)
Weil felix positives	19	44	63	78.5%	84.85%	30.16%	94.05%	84.4%
Weil felix negatives	04	365	369					
Total	23	409	432					

5.14. Performance characteristics of IgM ELISA and Weil–Felix test with titre > 1:320 in detection of Scrub typhus specific antibodies in serum samples.

Increase in the specificity and PPV was observed with the increase in the cut off titre 1:320 and above. Samples with titre of 1:160 were excluded here.

Table- 17: Performance characteristics of IgM ELISA and Weil–Felix test with titre > 1:320 in diagnosis of Scrub typhus specific antibodies in serum samples. (n 384)

Tests	ELISA Positiv	ELISA Negatives	Total	Sensitivity	Specificity	PPV	NPV	Accuracy
Weil felix positives	12	3	15	77%	94.5%	71%	99.5%	93.1%
Weil felix negatives	04	365	369					
Total	16	368	384					

5.15. Comparison of Clinical parameters with serological tests.

5.15.1. Comparson with Weil felix test

Clinical signs and symptoms were compared with the results obtained in weil felix test. Fever was the common symptom in all the cases. In seropositive cases, common signs and symptoms observed along with consistent fever were head ache, myalgia, nausea and vomiting. Major clinical sign, rash was observed in 29.5% of Weil felix positive cases. Four cases had serious complications.

Table 18: Comparison of clinical parameters with Weil felix test (n=572)

Signs and Symptoms	Positive cases (%)	Negative cases
Fever	226 (100)	346 (100)
Rash	63 (29.57)	05 (1.39)
Head ache	76 (35.68)	21 (5.57)
Myalgia	29 (13.61)	16 (4.45)
Edema	18 (8.45)	0 (0)
Eschar	0 (0)	0 (0)
Lymphadenopathy	6 (2.81)	2 (0.55)
Nausea	51 (23.94)	9 (2.50)
Vomiting	23 (10.79)	11 (3.06)
Other serious complications	4 (1.87)	0 (0)

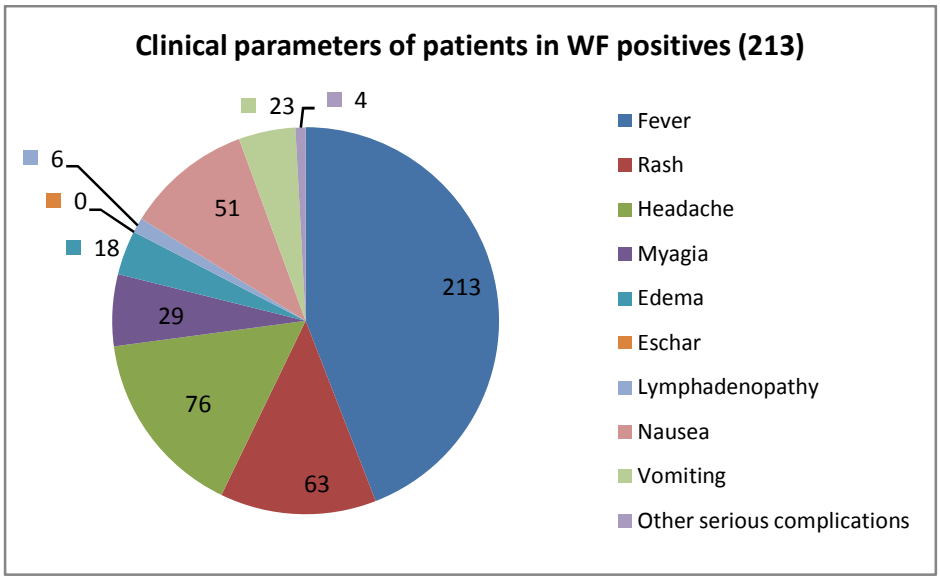


Fig-26. Comparison of Clinical parameters of patients in WF positives(n=213)

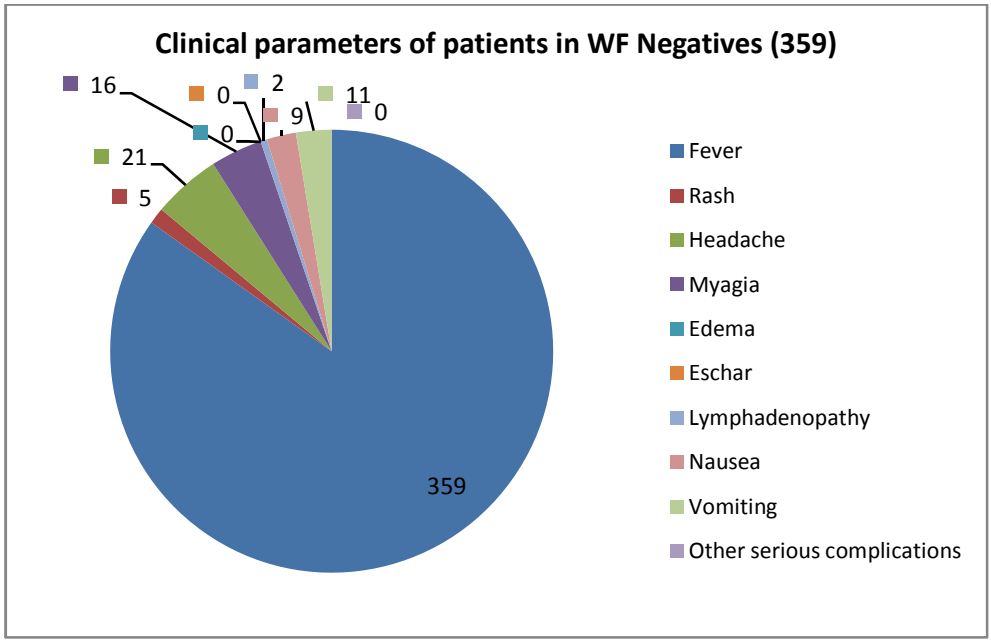


Fig-27. Comparison of Clinical parameters of patients in WF Negatives (n=359)

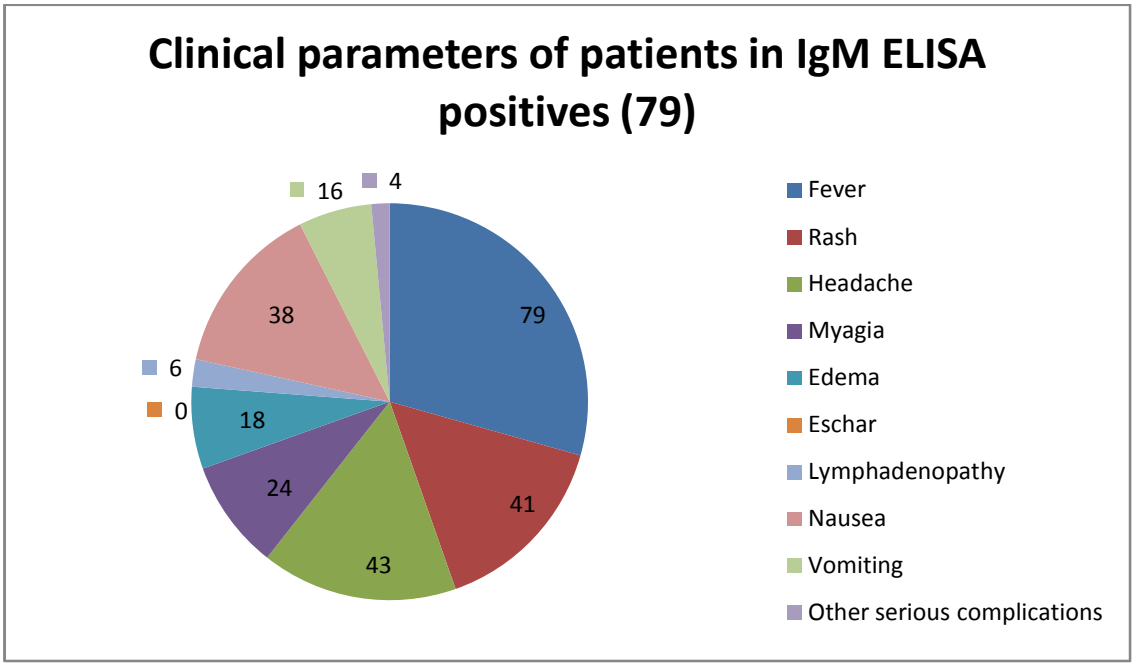


Fig-28. Comparison of Clinical parameters of patients in IgM ELISA positives

5.17. Molecular Characterization

Entire molecular work was carried out at NITM, ICMR, Belgaum under the guidance of Dr. S.L. Hoti, Scientist 'G'.

5.17.1. DNA extraction from blood clots of ELISA positive patients.

DNA extraction was done using QIAmp blood mini kit (Qiagen, USA) as per manufacturer's instructions.

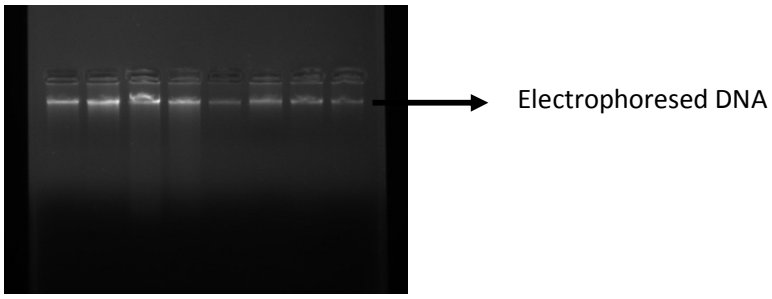
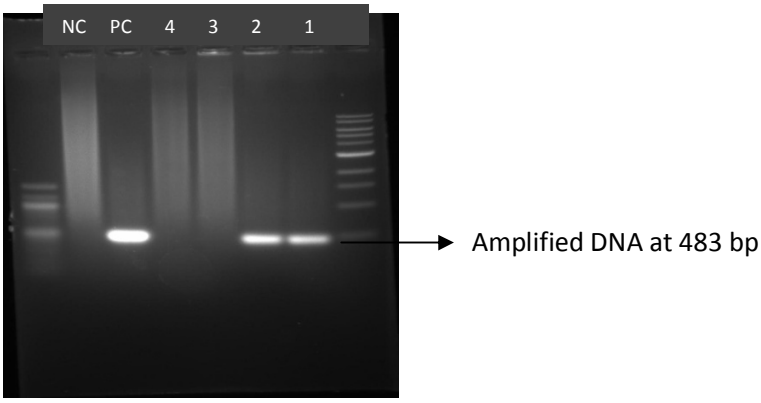


Fig-29. Electrophoresis on 1% agarose gel showing Electrophoresed DNA

5.17.2. Molecular detection of Scrub typhus by n-PCR.

In n-PCR for scrub typhus, a 483-bp fragment of the gene encoding 56-kDa TSA of *O. tsutsugumushi* was amplified using overlapping primers 10 and 11 as described Furuya et al. One known positive DNA and distilled water was used as positive and negative controls respectively. Upon request, the positive control was obtained from Dr. Sneha, Asst. Professor, Dept. of Microbiology, BMCRI, Bangalore



Lane 1- Sample 501, Lane 2- Sample 503, Lane 3- Sample 529, Lane 4- sample 487, Lane 5- Positive control, Lane 6- Negative control, Lane 7 – 100bp ladder.

Fig-30. Gel picture showing amplification of 483-bp fragment gene encoding 56-kDa TSA of *O. tsutsugumushi*.

5.17.2.1. DNA Sequencing:

Purification and sequencing of the PCR product was done at Eurofins genomics India Pvt. Ltd, Bangalore. PCR products were purified using a QIAquick gel extraction kit (Qiagen, USA) and sequenced bidirectionally using Applied Biosystems model 3730xl/3730XLPA-19137-17. Sanger dideoxy sequencing technology was used for the sequencing of DNA.

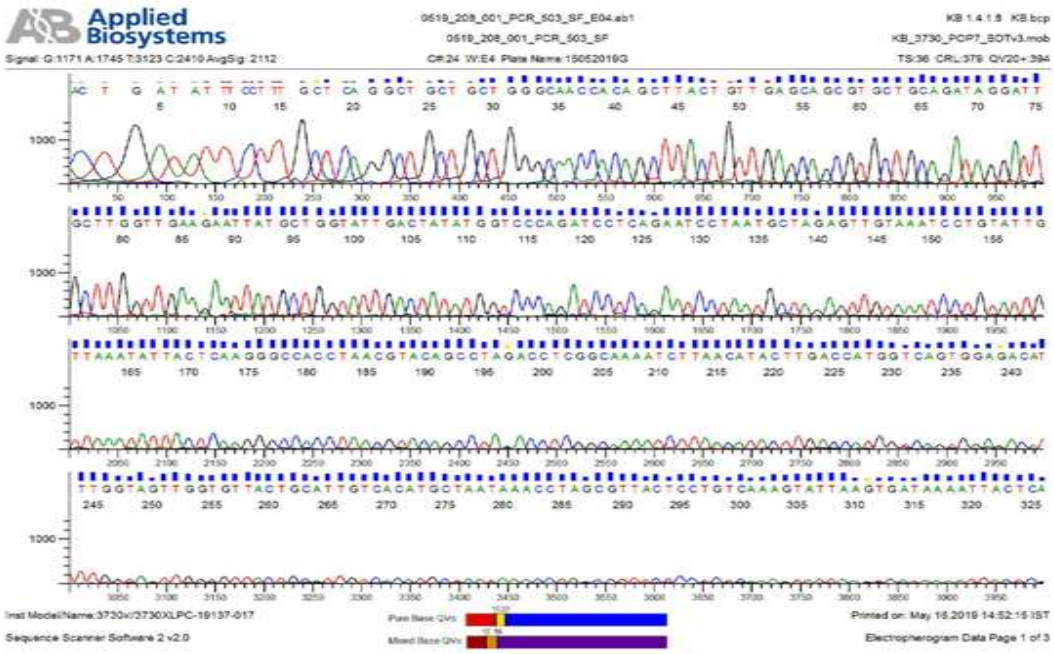


Fig-31. The chromatogram file of the sequenced sample showing good quality reads (sample No 503)

5.17.2.2. Checking the quality of sequenced samples

The Ab 1 files of sequenced samples were opened with Finch TV Version 1.4 to check the quality of sequenced samples.

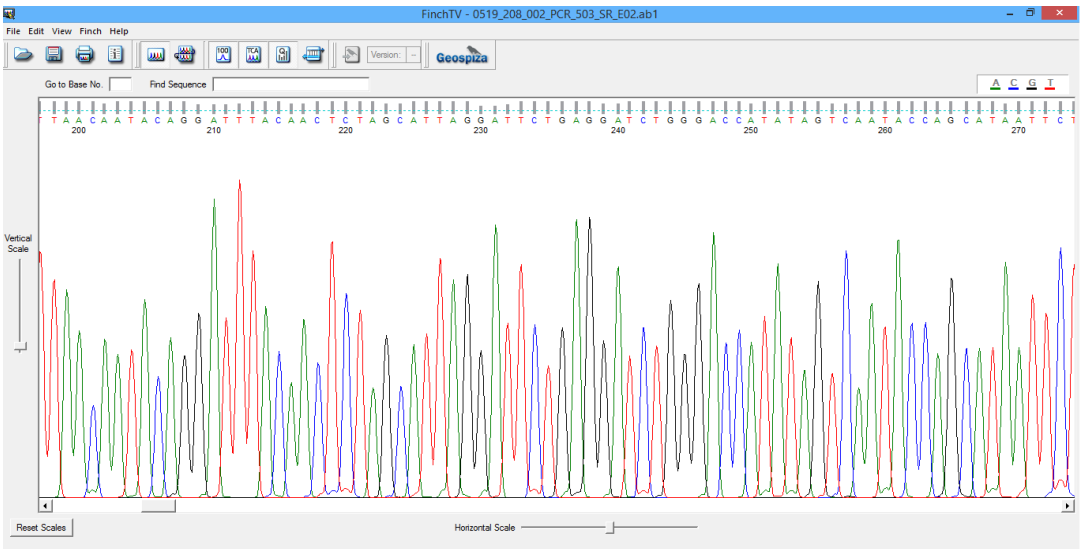


Fig-32: Quality check of sequenced samples using Finch TV Version 1.4

5.17.2.3. Generating consensus sequences

DNA Dragon V1.5.61 was used to generate consensus sequence

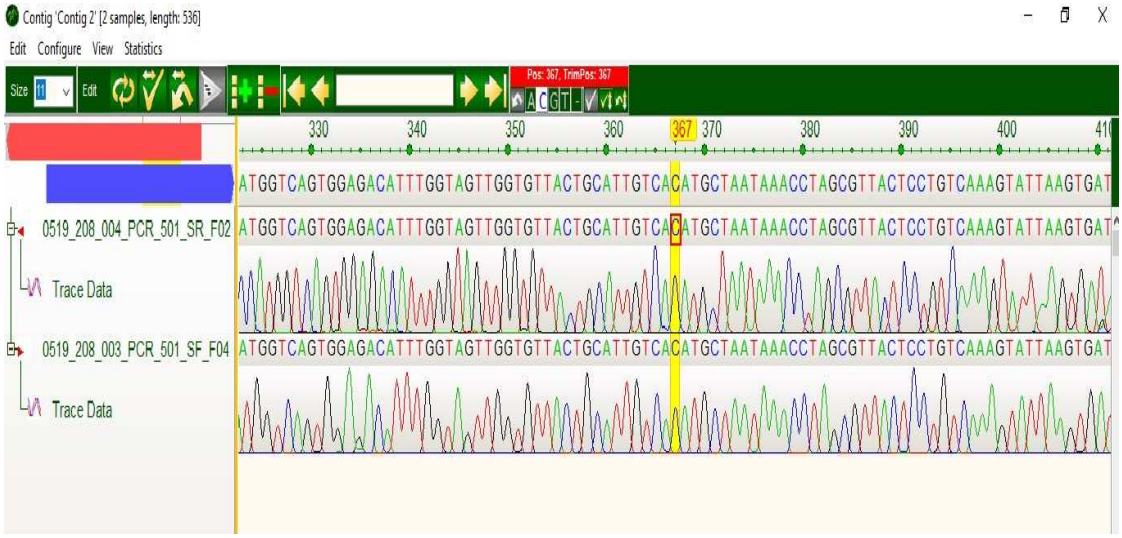


Fig-33: Generating consensus sequences using DNA Dragon V1.5.61

5.17.2.4. BLAST n analysis of sequence obtained in the study.

Sequences obtained in this study were blasted using NCBI BLAST to confirm the amplification of 483bp segment of the gene encoding 56kDa antigen of *Orientia tsutsugumushi*. Further, sequences obtained in our study were compared with the sequences reported from other places. BLASTn result revealed that sequences of this study had closest homology to maximum number the strains reported from India, and shown closest homology with some of the strains reported from other countries also like China, Vietnam, Cambodia, Taiwan, Bangladesh, Srilanka and few strains identified throughout Southeast Asia.

ACTGATATTTCCTTTGCTCAGGCTGCTGCTGGGCAACCACAGCTTACTGTTGAGCAGC
GTGCTGCAGATAGGATTGCTTG
GTTGAAGAATTATGCTGGTATTGACTATATGGTCCCAGATCCTCAGAATCCTAATGCTA
GAGTTGTAAATCCTGTATTGT
TAAATATTACTCAAGGGCCACCTAACGTACAGCCTAGACCTCGGCAAAATCTTAACATA
CTTGACCATGGTCAGTGGAGA
CATTTGGTAGTTGGTGTTACTGCATTGTCACATGCTAATAAACCTAGCGTTACTCCTGT
CAAAGTATTAAGTGATAAAAT
TACTCAGATATATAGGGATATAAAGCCATTTGCTGATATAGCTGGTATTAATGTTCCTGA
TACTGCTTTGCCTAATAGTG
CATCTGTCGTGGTGATTTAGAAGATTATTGCTATTAGCATGCCGCTTGGGGGGGGGA
GTCACCCCCCCCAGGGGGTTTAA
ACAGGGGCTGCAGGAGCGCTTGGCGCTGGGGGGAGGGGGGGAGGGGGGGGGGGGGGG
GCGGAAGGAG

Fig-34: Sequences obtained for the study sample No. 503.

Table- 19. BLAST NCBI result of Sequences obtained from the amplified DNA of gene encoding 56kDa antigen of Scrub typhus

Sl.No	Accession No.	Description of Top Match	Length	Coverage	Identity	Reported place
1	MF457892.1	Orientia tsutsugamushi isolate 201606239412 outer membrane protein gene, partial cds	423	74%	99.27%	India
2	MG283201.1	Orientia tsutsugamushi isolate KMC0700 56 kDa type specific antigen gene, partial cds	436	74%	99.27%	India
3	KT970967.1	Orientia tsutsugamushi isolate SS3187 56 kDa type-specific antigen gene, partial cds	427	74%	99.27%	India
4	DQ286233.1	Orientia tsutsugamushi clone ISS-11 56 kDa type specific antigen gene, partial cds	460	74%	99.27%	India
5	KR706188.1	Orientia tsutsugamushi isolate OT/MCVR/814203GOP/2014 56 KDa Type-specific antigen (tsa56) gene, partial cds	405	71%	100.00%	India
6	KX130950.1	Orientia tsutsugamushi strain GKP-R102 56 kDa type-specific antigen (tsa56) gene, partial cds	401	73%	99.25%	India
7	KR706186.1	Orientia tsutsugamushi isolate OT/MCVR/814019GOP/2014 56 KDa Type-specific antigen (tsa56) gene, partial cds	401	70%	100.00%	India
8	KT735174.1	Orientia tsutsugamushi isolate pgi24hp 56 kDa type-specific antigen gene, partial cds	389	69%	100.00%	India
9	MF197396.1	Orientia tsutsugamushi strain GKP134 56 kDa type specific antigen gene, partial cds	415	70%	99.22%	India
10	LS398551.1	Orientia tsutsugamushi str. Gilliam genome assembly, chromosome: I	2465012	74%	96.82%	United Kingdom
11	KY320040.1	Orientia tsutsugamushi isolate F12P 56 kDa protein gene, partial cds	479	74%	96.82%	China
12	KT970975.1	Orientia tsutsugamushi isolate SS1220 56 kDa type-specific antigen gene, partial cds	427	69%	99.21%	India

13	KT970970.1	Orientia tsutsugamushi isolate SS3456 56 kDa type-specific antigen gene, partial cds	430	69%	99.21%	India
14	KM115578.1	Orientia tsutsugamushi strain Sanjie3 56 kDa outermembrane protein gene, partial cds	685	74%	96.82%	China
15	JX976614.1	Orientia tsutsugamushi strain Hefei 56 kDa type-specific antigen gene, partial cds	1320	74%	96.82%	China
16	GU120147.1	Orientia tsutsugamushi strain KM02 56-kDa type-specific antigen (tsa56) gene, complete cds	1575	74%	96.82%	Taiwan
17	DQ514319.1	Orientia tsutsugamushi strain Neimeng-65 56 kDa type-specific antigen gene, partial cds	1535	74%	96.82%	China
18	KT970972.1	Orientia tsutsugamushi isolate SS172 56 kDa type-specific antigen gene, partial cds	427	68%	99.20%	India
19	DQ514322.1	Orientia tsutsugamushi strain Neimeng-88 56 kDa type-specific antigen gene, partial cds	438	74%	96.58%	China
20	KR706187.1	Orientia tsutsugamushi isolate OT/MCVR/814066GOP/2014 56 KDa Type-specific antigen (tsa56) gene, partial cds	375	66%	100.00%	India
21	DQ485289.1	Orientia tsutsugamushi isolate Taiwan 56kDa type-specific antigen gene, complete cds	1860	74%	96.33%	Taiwan
22	KT957880.1	Orientia tsutsugamushi clone PGI86HAR 56 kDa outer membrane protein gene, partial cds	366	65%	100.00%	India
23	KT957878.1	Orientia tsutsugamushi clone PGI57HAR 56 kDa outer membrane protein gene, partial cds	359	65%	100.00%	India
24	KT970965.1	Orientia tsutsugamushi isolate ISE560 56 kDa type-specific antigen gene, partial cds	418	67%	99.18%	India
25	HQ718421.1	Orientia tsutsugamushi clone S0617100_KH 56-kDa type-specific antigen gene, partial cds	1558	74%	95.84%	Cambodia
26	KT970977.1	Orientia tsutsugamushi isolate SS1226 56 kDa type-specific antigen gene, partial cds	409	65%	99.16%	India

27	KX185420.1	Orientia tsutsugamushi isolate eschar 12 56 kDa protein gene, partial cds	420	70%	96.14%	Srilanka
28	KT957877.1	Orientia tsutsugamushi clone PGI30PUN 56 kDa outer membrane protein gene, partial cds	377	68%	96.81%	India
29	EF213085.1	Orientia tsutsugamushi strain FPW2016 56-kDa type-specific antigen gene, complete cds	1608	74%	94.59%	Thailand
30	KY971315.1	Uncultured Orientia sp. clone YNYS20 56 kDa type-specific antigen gene, partial cds	744	74%	94.35%	China
31	KJ742363.1	Orientia tsutsugamushi strain TH4016 56 kDa protein gene, partial cds	892	74%	94.35%	Throughout Southeast Asia
32	MK617206.1	Orientia tsutsugamushi isolate OtMMC117 56-kDa type-specific antigen gene, partial cds	698	74%	93.89%	Bangladesh
33	KJ094995.1	Orientia tsutsugamushi clone SVU/SVIMS 28 56kDa type-specific antigen gene, partial cds	481	74%	93.89%	India
34	GU128874.1	Orientia tsutsugamushi strain Linh.DT No37 56kDa type-specific antigen gene, partial cds	475	74%	93.89%	Vietnam
35	MH570198.1	Orientia tsutsugamushi isolate Bangalore-13 56kDa type-specific antigen gene, partial cds	413	74%	93.87%	India
36	GQ260638.1	Uncultured Rickettsia sp. clone cmc08 56 kDa antigen gene, partial cds	472	74%	93.87%	India
37	KJ742371.1	Orientia tsutsugamushi strain S072 56 kDa protein gene, partial cds	919	74%	93.86%	Asia
38	EF213099.1	Orientia tsutsugamushi strain UT329 56-kDa type-specific antigen gene, complete cds	1596	74%	93.86%	THAILAND
39	AY834393.1	Orientia tsutsugamushi strain Hualien-7 56-kDa type-specific antigen gene, complete cds	1857	74%	93.61%	Taipei
40	KY594247.1	Orientia tsutsugamushi isolate RAS14231TN16 56 kDa outer membrane protein gene, partial cds	451	74%	93.60%	India

5.17.2.5. Phylogenetic analysis based on 56-kDa TSA of *Orientia tsutsugamushi* with sequences of the study

Phylogenetic analysis was performed with two DNA sequences obtained in the present study with the some of the established genotypes and reference strains retrieved from NCBI database.

5.17.2.6. Aligning the sequences obtained in the study

For the construction of Phylogenetic tree, sequences obtained in the study were aligned with reference sequences of *Orientia tsutsugamushi* using MEGA 7 software.

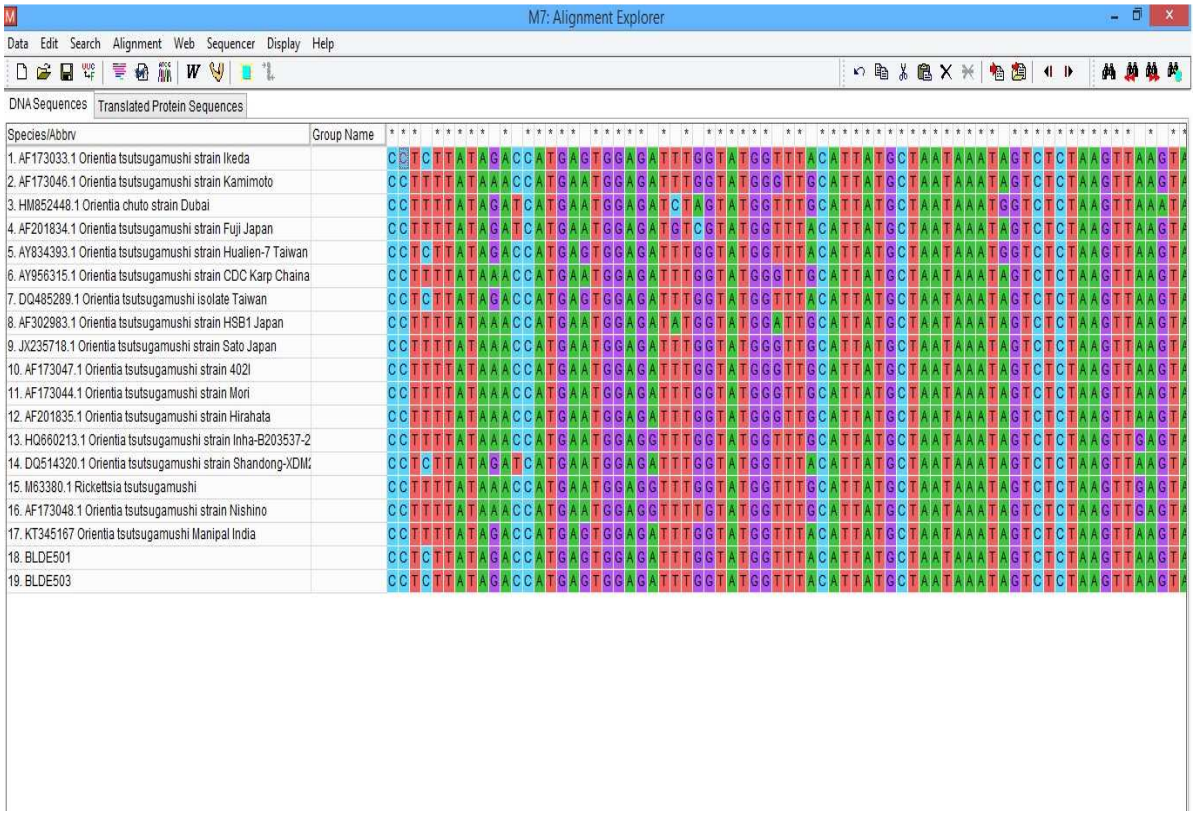


Fig-35: Aligning of sequences with reference sequences of *Orientia tsutsugamushi* in MEGA 7 software

5.17.2.7. Construction of Phylogenetic tree

Phylogenetic analysis was performed with two DNA sequences obtained in the present study with the some of the established genotypes and reference strains retrieved from NCBI database. Phylogenetic analysis of *Orientia tsutsugamushi* showing close relatedness of two isolates of our study (BLDE 501 and BLDE 503) with the strain Inha (Gilliam) reported from Inha, Korea.

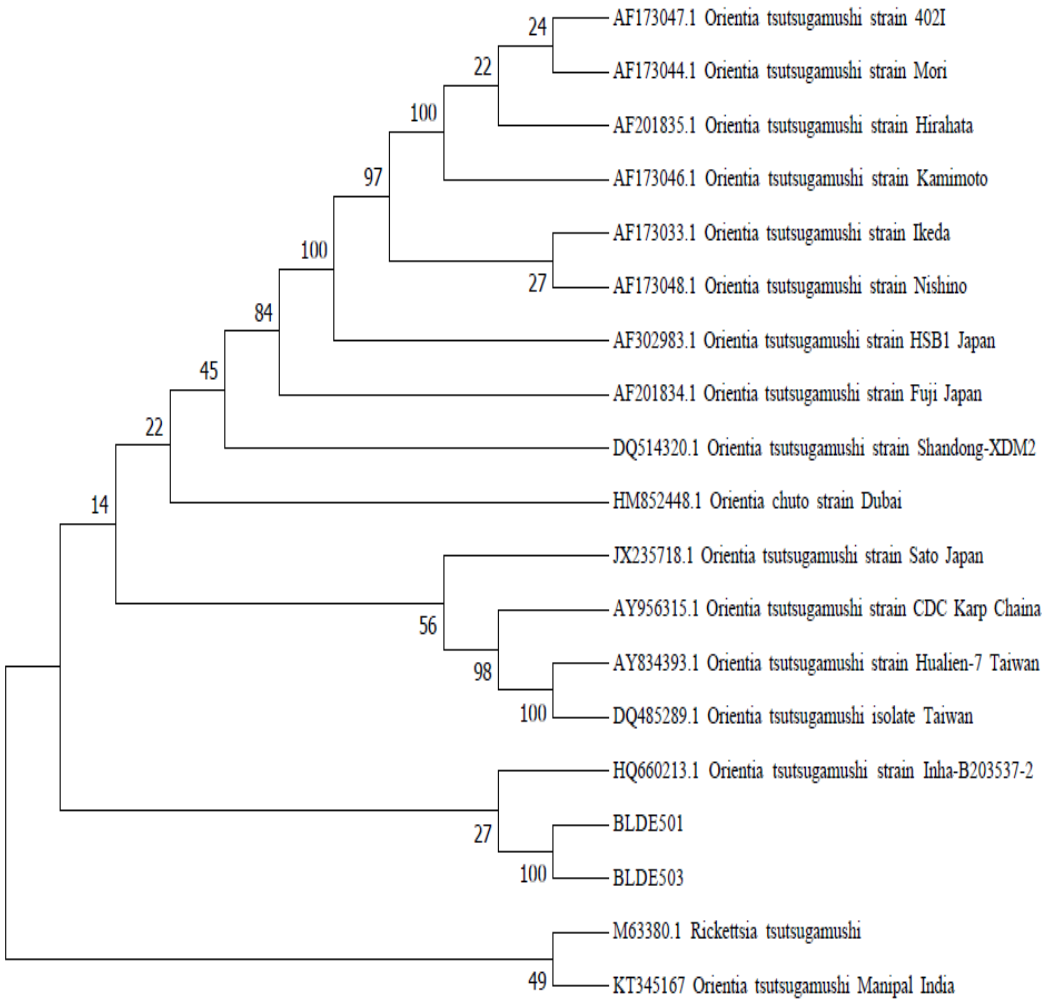


Fig-36: Phylogenetic analysis of *Orientia tsutsugamushi* isolates.

5.17.3. Molecular detection of SFG Rickettsiae by PCR

DNA was extracted from *Rickettsia conorii* ELISA positive blood clot samples. As no positive control was available, we have made several attempts to standardize the nPCR for the amplification of *R. conorii* DNA by changing the reaction conditions, applying gradient PCR etc. The primers used for the standardizing process were to detect a 382bp segment of the *Rickettsia* genus specific gene *gltA* (citrate synthase gene) and a 532bp segment of the SFGR specific outer membrane protein gene (*ompA)*. nPCR was performed using the same protocol and conditions as described by JAJ Prakash *et al.*[248] In spite of multiple attempts and changing reaction conditions, we could not standardize the PCR for the detection of *R. conorii* from blood clots.

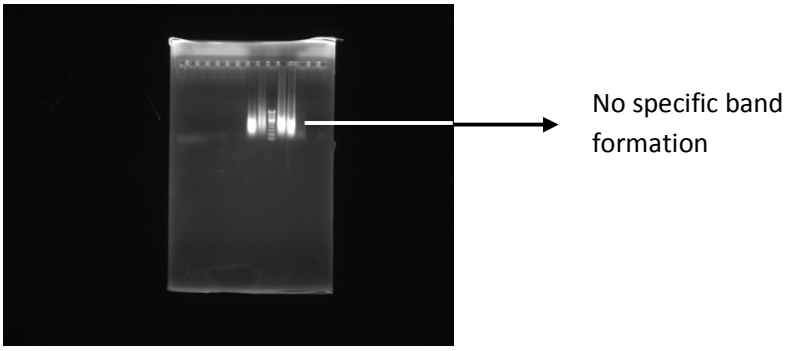


Fig- 37. Gel picture of unsuccessful amplification of the gene *ompA*. No amplification observed at desired basepair region.

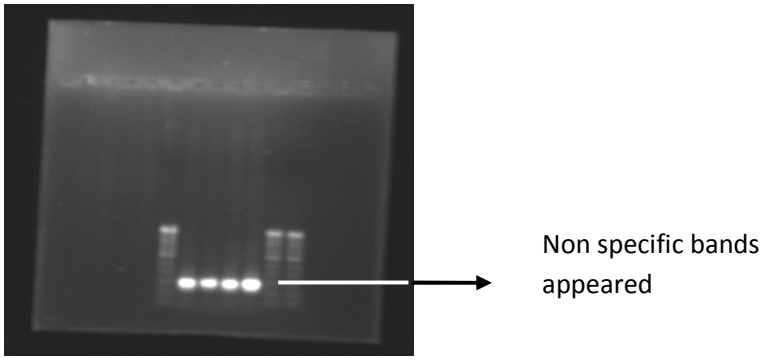


Fig. 38. Gel picture of unsuccessful amplification of the gene *gltA*. Non specific bands appeared, and no amplification observed at desired base pair region.

DISCUSSION

CHAPTER- 06

DISCUSSION

In India, Rickettsiosis is considered as one of the re-emerging infectious diseases of public health importance currently.[1] The decision for the diagnosis of rickettsial infection is generally made by the patients's history and clinical presentation. However, the vast variability and non specific clinical manifestations of the disease makes the clinical diagnosis challenging and may lead to misdiagnosis.[23,29,227,321]. Even now, accurate and early diagnosis of rickettsial infection remains a challenge because of its nonspecific presentation and the nonavailability of confirmatory diagnostic tests.[22] Timely diagnosis is essential since antibiotic therapy provides greatest benefit when initiated early in the course of illness.

Since last two decades, several researchers have reported the serological evidence of rickettsial infection from different geographical parts of India using single or a combination of serological tests like Weil felix, ELISA and Microimmunofluorescence assay[2-10,12,14-24,27-31,34,314-323] and using both serological test and molecular test- PCR[32,239, 248, 295, 296].

Though many cases of rickettsial infections were reported from southern part of Karnataka [23,48,296,323] the disease was not evaluated and no confirmatory data available from north Karnataka region. Moreover, it was observed that many cases were reported as positive for rickettsial infections in some of the laboratories in Vijayapura on the basis of single serological test Weil felix. Hence, we undertook this study to investigate the existence of Rickettsial infection in and around Vijayapura area of North Karnataka

region using serological methods and confirmation of etiological agent of the disease circulating in this region by molecular diagnostic test PCR.

Demographic data

In this study we have screened 572 blood samples collected from the patients of all age groups and both the sexes presenting with acute febrile illness and the signs and symptoms of Rickettsial infection. Patient history, clinical presentations and demographic data were collected and recorded.

Age: In this study the age of the patients from which blood samples were collected ranges from 1 year to 74 years. Age wise distribution of cases demonstrated significantly high number of cases in the age groups between16-30 (32.69%) and 46 and above (29.89%) including both male and females (Table 5).

Gender: Out of 572 cases, there was a clear preponderance of male patients across all age groups with 63.46% (363) and female cases were 36.53% (209). Same scenario was reported in a study by Sneha *et al* from Bangalore.[23] In contrary, equal distribution of the cases also reported by some of the researchers. Chaudhury *et al* from Haryana have reported 52% male and 48% female.[6]

Weil felix test:

As per the study protocol, all the samples were screened for the evidences of seropositivity for Rickettsial infection by the widely used serological test i.e. Weil felix tube agglutination test. Samples shown agglutination at titre of 1:160 and above with OX2, OX19 and OXK antigens were considered as positive. Out of 572 cases 213 (37.23%) samples have shown titre of 1:160 and above with OX antigens. In 213 WF positive cases, 180 (31.46) samples have shown agglutination with OX2 antigen alone or

with other antigens suggestive of SFG Rickettsial infection and 63 (11.01%) cases shown agglutination with OXK antigen alone or with other antigens suggestive of Scrub typhus infection (Table 6). Results obtained in our study for SFG Rickettsiae shows comparatively higher positivity in comparison with similar studies reported by other researchers. In a study from Delhi by Veena Mittal *et.al* have reported 27.5% positivity for SFGR[318] and Raghu Kumar *et.al* from Davanagere have reported the positivity of 23.3% for SFG Rickettsiae by Weil felix test[323]. The 63 (11.01%) cases with OXK 1:160 and above suggestive of Scrub typhus is highly significant in this region even though the results of our study shows considerable difference in percentage of positive cases reported by several researchers from different geographical parts of India like Karnataka[23,316] , Andrapradesh[317,322], Pondicherry[18], Tamil Nadu[34], Himachal Pradesh[256] and Delhi.[318]

Age and gender wise distribution of positivity by Weil felix test in our study demonstrated slightly higher seropositivity in males and in the age group of 16 to 30 and 46 and above (Table 7). Findings of this study concur with many similar data reported by other researchers. Kamarasu *et.al* reported positivity of 66% in male during the year 2004, and 53% in 2005[34]. Raghukumar *et al* have reported male preponderance of 57% in their study. [323] That might be due to exposure to vectors when they go out to the fields. On contrary, Meerah *et.al* from Tamil Nadu reported more positivity in female i.e. 65%[315], our findings shows meager disparity with this.

Seasonal variation:

In the present study, significantly higher number of cases and seropositivity was observed during the cooler months i.e. between August to January every year (Table 8). Similar scenario was observed and reported by many of the researchers in their study.[23,34,137, 318, 320.] The increased number of cases in the cooler months may be directly attributed to the increased vectors activities, as temperature is known to play an important role in the activity of vectors and their life cycle.

IgM ELISA:

For the serological confirmation we have screened all the samples further by another serological test IgM ELISA (Table 10&11). IgM ELISA was proved to be highly sensitive and specific in some of the studies carried out by other investigators during last 10 – 15 years. Munegouda *et.al* from Karnataka reported sensitivity and specificity of 83% and 95% respectively[316]. Meerah *et.al* from Tamil Nadu have reported sensitivity and specificity of 96.7% and 98.18% respectively.[315] Out of 572 cases subjected to SFG specific IgM ELISA, 56 samples confirmed found to be seropositive. Among 56 positives, 45 samples were positive by WF test also, and 11 samples were negative by WF test. (Table 14&15) Out of 432 samples, 23 found to be seropositive by IgM ELISA for Scrub typhus. Among 23 ELISA positives, 19 samples were positive by WF test also, and 4 samples were negative by WF test (Table 16 &17). We have obtained more false positives by Weil felix test and it shows comparatively high disparity with most of the researchers as they have observed more positivity by ELISA than weil felix test. In a study by Roopa *et.al* from Puducherry have reported more positivity by ELISA than

WF[29]. The false negatives might be due to early stage of disease as the agglutinating antibodies can be detected only in the second week of illness[318].

In this study the seropositivity rate for both SFG Rickettsiae and Scrub typhus by both ELISA and WF is highly significant with values 31.46% and 9.79% for SFGR, which is in concordant with the report from 11.01% and 5.32% for Scrub typhus (Table 12&13), agrees with report from other researchers also.[29,315]

Though the clinical manifestation play major role in the diagnosis of disease, it is difficult to make the decision clinically due to vast variability and common clinical manifestations of the disease which is similar to other febrile illnesses like dengue, malaria etc. Clinical features seen in our study are, fever in all the cases (100%), rash (29%), headache (35%), myalgia (13.6%), Nausea (24%), vomiting (11%), edema (8.5%) nausea and 4 cases were admitted with serious complications (Table 18). Our results are concurrent with other investigators with respect to percentage of signs and symptoms in positive and negative cases. Somashekar *et al*,[227] sneha *et al*,[23] Mahajan *et al*,[256] and Anita *et al* [295] We could not find/ assess any cases with eschar as it is mostly seen in scrub typhus cases than SFGR infection[49,229,232].

We have observed better Sensitivity and specificity for Weil felix test in comparison with ELISA (Table14&16), increase in cut off titre i.e. 1:320 and above showed much better specificity and PPV (Table 15&17). Result obtained in our study showed meager disparity with similar studies reported by other investigators. Munegouda *et.al* from Karnataka reporteded that cutoff titre of 1:160 for Weil felix increases the sensitivity[316], in another study by Seemarani *et.al* from Himachal Pradesh reported

increase of increase of specificity up to 100% when increased the titre and sensitivity was decreased[314].

Since both the serological tests exhibited significant variability in sensitivity and specificity in the reports from different geographical area[10,23,29,227,316-323], the detection of causative organism directly from the patients sample would be highly important and confirmatory. Different PCR techniques have been reported during the last decade for detection of different genes specific to *Rickettsia* spp.[248,298,301,307,311] and *Orientia*.[239,293,295-296,299-301,310,312]

To substantiate the seropositivity observed in the study area for Scrub typhus, we performed the nested PCR on blood clots of ELISA positive samples for the confirmation of etiological agent using the primers specific to the gene encoding 56kDa antigen of O. *tsutsugumushi* as described by *Furuya et.al*. While ELISA positive samples had high OD value, we succeeded to amplify a 483 bp segment of the gene encoding the 56 kDa antigen of *O. tsutsugamushi* in only two samples which were positive by both the serological tests Weil Felix and IgM ELISA. Results of Nucleotide sequences obtained for our samples were blasted using NCBI BLAST to confirm the amplification of the DNA of gene encoding 56kDa antigen of *Orientia tsutsugumushi*.[309] Further, sequences obtained in our study were compared with the sequences reported from other places. BLASTn result revealed that sequences of our study had closest homology to maximum number the strains reported from India, and shown closest homology with the strains reported from other countries also like China, Vietnam, Cambodia, Taiwan, Bangladesh, Srilanka and few strains identified throughout Southeast Asia. (Table-19)

Phylogenetic analysis was performed with two DNA sequences obtained in the present study with the some of the established genotypes and reference strains retrieved from NCBI database. For the construction of Phylogenetic tree, sequences obtained in the study were aligned with reference sequences of Orientia tsutsugamushi using MEGA 7 software. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 13.31558833 is shown. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 19 amino acid sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position.

The circulating genotypes of *O. tsutsugamushi* in India is not clearly known or reported except few studies. S.K Mahajan *et al.*[21] from Himachal Pradesh have reported the genotypes of *O. tsutsugamushi* for the first time in India on the basis of phylogenetic analysis. They reported ≥ 2 different genotypes in their study, one between Karp and JP-1 cluster and the other between Saitama and JG type cluster. In another study by Varghese *et al.*[163] from Tamil Nadu have reported 26 sequences in their study. Seventeen of the 26 strains (65%) were clustered under the Kato group, 8 (35%) in Karp like isolates, and only 1 with the Gilliam strain. Another study by Munegowda *et al*. from Karnataka has reported an isolate clustered close to genotype Ikeda.

We made several attempts to amplify the SFGR specific DNA for identification of genus and species by using the specific primers gltA, ompA as described by JAJ Prakash et. al from India, Ishikura et al from Japan. In spite of multiple attempts and changing

reaction conditions, we could not standardize the PCR for the detection of *R. conorii* from blood clots. It may be due to collection of the samples in the delayed stage of the illness and long storage of the blood clots.

SUMMARY & CONCLUSION

CHAPTER- 7

SUMMARY AND CONCLUSION

Summary

- Rickettsial infections, transmitted by the infected arthropod vectors are the most covert re emerging febrile infection in the current era. The disease is unnoticed or misdiagnosed due to low manifestation and lack of specific diagnostic tests at all levels.
- Geographically the disease is widely endemic, prevalent in various parts of the world and in India. Our study demonstrated that in Vijayapura region predominant cases were suggestive of Spotted Fever Group Rickettsiae than Scrub typhus in both Weil felix test and IgM ELISA.
- All the patients subjected in the study were presented with acute febrile illness, and in most of the seropositive cases headache, myalgia, nausea, vomiting and edema were the common clinical signs and symptoms. Rashes were observed in significant number of cases with SFGR seropositive cases. The result demonstrated that clinical diagnosis is most important for making the decision towards diagnosis of Rickettsial infection.
- More number of suspects and comparatively high seropositivity was observed during the colder season between august to January every year.
- Male preponderance in distribution and seropositivity was observed in all age groups. Higher seropositivity was observed in the age groups between 16 to 30 and 46 & above. This might be due to frequent exposure to the vectors in the field.

- In the present study we have observed comparatively improved sensitivity and specificity for WF test in comparison with ELISA with the increased cutoff titre value to 1:320. As we have observed more number of false positives and a few false negatives, inclusion of ELISA in routine diagnostic protocol is highly necessary.
- Though the molecular diagnosis method PCR proved to be more specific and sensitive by other reasearchers, we could not confirm SFG Rickettsiae from blood clot samples even though cases were clinically suspected and serologically positive, needs to be standerdized. Reason might be the clearance of pathogen by the host immune system at the time of sample collection and long storage of the samples.
- Two *O. tsutsugumushi* isolates sequenced in the study was showing 95to 100% homology with the several strains reported from various parts of India and Southeast Asia. Phylogenetic analysis of the sequences was showing close relatedness with the Gilliam strain reported from Korea (Inha).
- Molecular detection of *O. tsutsugumushi* in the present study has proved the presence Scrub typhus disease in and around Vijayapura. This the first report from this region.
- As establishment of the molecular level laboratory at all levels is not feasible, inclusion of more sensitive and specific test like ELISA would be better alternative.

Conclusion

Currently, Rickettsiosis is one of the re-emerging febrile infections across the world. But due to its non specific clinical feature, diagnosis of this disease is challenging, and leads to misdiagnosis in most of the time. Findings of our study clearly demonstrated that Rickettsial infections, both Spotted fever and Scrub typhus are also circulating and causing acute febrile illness among the populace in and around Vijayapura. Significant seropositivity was observed for SFGR than Scrub typhus; however molecular detection of scrub typhus has confirmed its existence too in this area. More seropositivity was observed during the cooler months (August to January). In the present study, the preliminary screening test WF has produced more number of false positives, and exhibited lack of specificity and sensitivity in comparison with IgM ELISA. However the performance of the WF test was improved with increase in cut off titre. With the strong clinical suspicion, Weil felix test remains to be a useful tool in a hospital setting where specific tests are not available. Inclusion of more specific serological test like IgM ELISA in the routine diagnostic course is highly beneficial for the timely diagnosis.

The two isolates we sequenced in the study is the first molecular evidence for scrub typhus from this region. Even though there was no amplification in the nPCR performed to detect SFGR, the high seropositivity observed in *R. conorii* IgM ELISA was the clear indication of the presence of SFGR infection in this region. Nested PCR is a rapid confirmatory test for the detection of pathogen from the samples during initial stage of the illness, hence it is recommended to employ PCR for the confirmation of the disease.

This study also helps the authorities to undertake therapeutic as well as preventive measures to prevent the morbidity and mortality due to rickettsiosis.

STUDY LIMITATIONS & FUTURE DIRECTIONS

CHAPTER-08

STUDY LIMITATIONS & FUTURE DIRECTIONS

Limitations:

- Non availability of positive control for standardization of PCR for SFG Rickettsiae.
- Though significant seropositivity was observed in IgM ELISA, only a few cases have been confirmed by molecular method. It might be due to collection of the samples in the delayed stage of illness and long storage of the blood clot samples.

FUTURE PROSPECTIVE

Rickettsial infection should be considered for the differential diagnosis of AFI, especially when associated with rash, edema, gastrointestinal symptoms, seasonality etc. More prospective studies with large number of samples need to be conducted on this neglected re-emerging illness to know the prevalence of the Rickettsial infections in this region.

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CHAPTER -09

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ANNEXURE

B.L.D.E. UNIVERSITY's SHRI B.M. PATIL MEDICAL COLLEGE HOSPITAL & RESEARCH CENTRE
BIJAPUR -586103

INFORMED CONSENT FORM

TITLE OF THE PROJECT : **Serodiagnosis and Molecular Characterization of Rickettsia in and around Bijapur, North Karnataka, India.**

PRINCIPAL INVESTIGATOR : **Mr. Shriharsha Hegde M.L.**
Research Scholar
Dept. of Microbiology.

PURPOSE OF THE RESEARCH (ಸಂಶೋಧನೆಯ ಉದ್ದೇಶ**):**

I have been informed that this study will be done to detect Rickettsial infections and to know the prevalence of Rickettsia.

Rickettsial ಸೋಂಕು ಪತ್ತೆಹಚ್ಚಲು ಮತ್ತು Rickettsial infection ಪ್ರಭುತ್ವವನ್ನು ತಿಳಿಯಲು ಈ ಅಧ್ಯಯನವನ್ನು ಮಾಡಲಾಗುತ್ತದೆ ಎಂದು ನನಗೆ ತಿಳಿಸಲಾಗಿದೆ.

PROCEDURE (ವಿಧಾನ**) :**

I understand that any clinical sample received from me will be subjected to various investigations needed for research purpose

ನನ್ನಿಂದ ಪಡೆದ ಯಾವುದೇ ವೈದ್ಯಕೀಯ ಮಾದರಿಯು ಸಂಶೋಧನಾ ಉದ್ದೇಶಕ್ಕಾಗಿ ಬೇಕಾದ ವಿವಿಧ ತನಿಖೆಗಳಿಗೆ ಒಳಪಟ್ಟಿರುತ್ತದೆ ಎಂದು ನಾನು ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ.

RISKS AND DISCOMFOPTS (ಅಪಾಯಗಳು ಮತ್ತು ಅಡ್ಡ ಪರಿಣಾಮಗಳು**):**

I understand that there is no risk involved in the procedures performed.

ನಡೆಸಿದ ಕಾರ್ಯವಿಧಾನಗಳಲ್ಲಿ ಯಾವುದೇ ಅಪಾಯವಿಲ್ಲ ಎಂದು ನಾನು ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ.

BENEFITS (ಲಾಭಗಳು**) :**

I understand that my participation in the study will help me providing appropriate treatment for my disease, as well as the researcher and Clinicians giving knowledge and comprehension in the early diagnosis and treatment of the Rickettsial infections.

ಅಧ್ಯಯನದಲ್ಲಿ ನನ್ನ ಪಾಲ್ಗೊಳ್ಳುವಿಕೆಯು ನನ್ನ ಕಾಯಿಲೆಗೆ ಸೂಕ್ತವಾದ ಚಿಕಿತ್ಸೆಯನ್ನು ಒದಗಿಸುವಲ್ಲಿ ನನಗೆ ಸಹಾಯ ಮಾಡುತ್ತದೆ, ಅಲ್ಲದೆ ಸಂಶೋಧಕರು ಮತ್ತು ಚಿಕಿತ್ಸಕರು Rickettsial ಸೋಂಕುಗಳ ಆರಂಭಿಕ ರೋಗನಿರ್ಣಯ ಮತ್ತು ಚಿಕಿತ್ಸೆಯಲ್ಲಿ ಜ್ಞಾನ ಮತ್ತು ಗ್ರಹಿಕೆಯನ್ನು ನೀಡುತ್ತದೆ.

CONFIDENTIALITY (ಗೌಪ್ಯತೆ)**:**

I understand that the medical information produced by the study will become a part of the hospital record and will be subjected to confidentiality and privacy regulations of the hospital. The researcher has given me the assurance that, if the data is used for publication my identity will not be revealed.

ಅಧ್ಯಯನದ ಮೂಲಕ ಉತ್ಪತ್ತಿಯಾದ ವೈದ್ಯಕೀಯ ಮಾಹಿತಿಯು ಆಸ್ಪತ್ರೆಯ ದಾಖಲೆಯ ಭಾಗವಾಗಲಿದೆ ಮತ್ತು ಆಸ್ಪತ್ರೆಯ ಗೌಪ್ಯತೆ ಮತ್ತು ಗೌಪ್ಯತೆ ನಿಬಂಧನೆಗೆ ಒಳಗಾಗುತ್ತದೆ ಎಂದು ನಾನು ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ. ಪ್ರಕಟಣೆಗಾಗಿ ಡೇಟಾವನ್ನು ಬಳಸಿದರೆ ನನ್ನ ಗುರುತನ್ನು ಬಹಿರಂಗಪಡಿಸಲಾಗುವುದಿಲ್ಲ ಎಂದು ಸಂಶೋಧಕರು ನನಗೆ ಭರವಸೆ ನೀಡಿದ್ದಾರೆ.

REQUEST FOR MORE INFORMATION (ಹೆಚ್ಚಿನ ಮಾಹಿತಿಗಾಗಿ ವಿನಂತಿ) :

I understand that I may ask more information about the study at any time. I will be informed of any significant new findings discovered during the course of study, which might influence my continued participation. A copy of this consent form will be given to me for my reference and careful reading.

ಯಾವುದೇ ಸಮಯದಲ್ಲಿ ನಾನು ಅಧ್ಯಯನದ ಬಗ್ಗೆ ಹೆಚ್ಚಿನ ಮಾಹಿತಿ ಕೇಳಬಹುದು ಎಂದು ನಾನು ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ. ಅಧ್ಯಯನದ ಸಮಯದಲ್ಲಿ ಕಂಡುಹಿಡಿಯಲಾದ ಯಾವುದೇ ಹೊಸ ಸಂಶೋಧನೆಗಳ ಬಗ್ಗೆ ನನಗೆ ತಿಳಿಸಲಾಗುವುದು, ಇದು ನನ್ನ ಮುಂದುವರಿದ ಭಾಗವಹಿಸುವಿಕೆಯನ್ನು ಪ್ರಭಾವಿಸುತ್ತದೆ. ನನ್ನ ಉಲ್ಲೇಖ ಮತ್ತು ಎಚ್ಚರಿಕೆಯಿಂದ ಓದುವ ಸಲುವಾಗಿ ಈ ಸಮ್ಮತಿಯ ರೂಪದ ಪ್ರತಿಯನ್ನು ನನಗೆ ನೀಡಲಾಗುವುದು.

REFUSAL FOR WITHDRAWAL OF PARTICIPATION (ಪಾಲ್ಗೊಳ್ಳುವಿಕೆಯ ನಿರಾಕರಣೆ) :

I understand that my participation is voluntary, and I may refuse to participate or may withdraw from the study at any time.

ನನ್ನ ಪಾಲ್ಗೊಳ್ಳುವಿಕೆ ಸ್ವಯಂಪ್ರೇರಿತವಾಗಿದೆ ಎಂದು ನಾನು ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ, ಮತ್ತು ಯಾವುದೇ ಸಮಯದಲ್ಲಾದರೂ ಅಧ್ಯಯನದಿಂದ ಭಾಗವಹಿಸಲು ಅಥವಾ ಹಿಂತೆಗೆದುಕೊಳ್ಳಲು ನಾನು ನಿರಾಕರಿಸಬಹುದು.

INJURY STATEMENT (ಗಾಯದ ಸಂಬಂಧಿತ ಹೇಳಿಕೆ) :

I understand that in the unlikely event of injury to me during the study, I will get medical treatment but no further compensations.

ಅಧ್ಯಯನದ ಸಮಯದಲ್ಲಿ ನನ್ನ ಗಾಯದ ಅಸಂಭವ ಘಟನೆಯಲ್ಲಿ, ನಾನು ವೈದ್ಯಕೀಯ ಚಿಕಿತ್ಸೆ ಪಡೆಯುತ್ತೇನೆ ಆದರೆ ಮತ್ತಷ್ಟು ಪರಿಹಾರವನ್ನು ಪಡೆಯುವುದಿಲ್ಲ ಎಂದು ನಾನು ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ.

I have read and fully understood this consent form. Therefore I agree to participate in the present study.

ನಾನು ಈ ಸಮ್ಮತಿ ಫಾರ್ಮ್ ಅನ್ನು ಓದಿದ್ದೇನೆ ಮತ್ತು ಸಂಪೂರ್ಣವಾಗಿ ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ. ಆದ್ದರಿಂದ ನಾನು ಪ್ರಸ್ತುತ ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸಲು ಒಪ್ಪುತ್ತೇನೆ.

_____

Participant/Guardian (ಪಾಲ್ಗೊಳ್ಳುವವರು / ಗಾರ್ಡಿಯನ್) Date:

_____

Signature of the witness (ಸಾಕ್ಷಿ ಸಹಿ) Date:

I have explained the patient the purpose of the study, the procedure required and possible risk and benefit to the best of my ability in the vernacular language.

ನಾನು ರೋಗಿಯನ್ನು ಅಧ್ಯಯನದ ಉದ್ದೇಶ, ವಿವರಿಸುವ ವಿಧಾನ ಮತ್ತು ಸಾಧ್ಯವಾದ ಅಪಾಯ ಮತ್ತು ಪ್ರಭಾವಿ ಭಾಷೆಯಲ್ಲಿ ನನ್ನ ಸಾಮರ್ಥ್ಯದ ಉತ್ತಮ ಪ್ರಯೋಜನವನ್ನು ವಿವರಿಸಿದೆ.

_____

Investigator (ತನಿಖೆದಾರ) Date:

_____

Witness signature (ಸಾಕ್ಷಿ ಸಹಿ) Date:

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Serodiagnosis and Molecular Characterization of Scrub Typhus in and Around Vijayapura, North Karnataka Region

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Abstract

Background: Scrub typhus or tsutsugamushi disease, transmitted by the bites of infected immature mites (chiggers) is a most covert re-emerging febrile infection currently. The disease is unnoticed or misdiagnosed due to low manifestation and lack of specific diagnostic tests at all levels. Failure of timely diagnosis leads to significant morbidity and mortality. Geographically this disease is widely endemic in a confined area of the Asia-Pacific region. In India, Scrub typhus infection is increasing and reported from various geographical parts during the past 10-15 yrs. Serological test is widely used for the diagnosis of the disease.

Aim & Objectives: To investigate the presence of Scrub typhus infection in and around Vijayapura of North Karnataka region using serological tests and nested PCR.

Materials and Method: During the period of 2015-17, a total of 209 patients presenting with acute febrile illness with rashes, body ache were examined for Scrub typhus infections by Weil Felix agglutination test and IgM ELISA. Further all the ELISA positive sampleswere tested by nested PCR using the specific primers of the gene encoding the immunodominant 56 kDa protein and PCR products were sequenced.

Results: Out of 209 cases, 39 (18.5%) samples showed agglutinationin Weil Felix antigen OXK test with titre 1:160 and above. In Scrub typhus IgM ELISA 13 (6.3%) samples were positive with the OD value more than 0.5. In nested PCR, two samples were amplified a 483 base pairs diagnostic segment of the gene encoding the 56 kDa antigen of *O. tsutsugamushi* using specific primers. PCR product was sequenced bidirectionally, and nucleotide sequences queried against NCBI BLAST programme to identify the sequenced sample.

Conclusion: Findings of our study demonstrated that, though more seropositivity of Spotted Fever Group Rickettsial infection is observed by WF test, scrub typhus infection is also circulating and causing acute febrile illness in and around Vijayapura, North Karnataka region. As the routinely used Weil felix test is less sensitive, inclusion of more specific tests like ELISA and nested PCR is very useful in proper diagnosis and patient management.

Keywords: *Weil Felix test, Rickettsia, Scrub typhus, ELISA, Nested PCR.*

Introduction

Rickettsial diseases are the most covert re-emerging febrile infections of the current era and mostly go unnoticed or misdiagnosed due to low manifestation and lack of specific diagnostic tests at all levels. Biogroups of Rickettsiaceae includes Spotted fever group, Typhus group and Scrub typhus[1]. Scrub typhus or tsutsugamushi disease is caused by the bacterium Orientia *tsutsugamushi*, which is transmitted by the

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bites of infected, immature mites (chiggers) belonging to the family Trombiculidae. Illness typically begins after the bite of an infected chigger and lasts for 7-10 days. The most common symptoms of infected persons are consistent fever of more than 7 days, headache, myalgia, rashes, eschar and lymphadenopathy. Delay in diagnosis and treatment leads to severe complications like central nervous system problems or circulatory collapse caused by disseminated intravascular coagulation and even death of the individual[2,3]. Geographically this disease is widely endemic in a confined area of the Asia- Pacific region, distributed in tsutsugamushi-triangle i.e. India and Nepal in the west; China, Japan, South Korea, and Taiwan in the north, and Australia and Indonesia in the south[2,3,4]. In India, the presence of Rickettsiosis has been reported from Jammu and Kashmir, Himachal Pradesh, Delhi, Rajasthan, Uttaranchal, West Bengal, Assam, Meghalaya, Karnataka, Andra Pradesh, Maharashtra, Tamil Nadu, Puducherry and Kerala[3-14].

During the last 10 - 15 years, several researchers from different geographical parts of India have reported seropositivity of scrub typhus infections using single or combination of serology based tests like, ELISA, Weil felix and Microimmunofluorescence assay etc[3-14]. Further, a few researchers have also demonstrated the PCR, a molecular test for the diagnosis of scrub typhus by detecting different genes, namely *56kDa*[15-20], *Groel*, and *16SrRNA*[16] from blood samples [15-19] and rodents [20].

When we reviewed the hospital medical records of last two years, we observed significantly high number of acute febrile illness cases diagnosed as Rickettsial infection on the basis of single serological test i.e. Weil felix. Though Scrub typhus infection has been increasing and reported from various geographical parts of India, no confirmatory data is available from north Karnataka region. Hence, we undertook this studyduring the period 2015– 2017 to investigate the existence of Scrub typhus infection in and around Vijayapur, Karnataka using both serological tests and molecular test- nested PCR.

Materials and Method

During the period of 2015-17, a total of 209 patients presenting with acute febrile illness, rashes and body ache were screened for the presence of Scrub typhus infection. It was a prospective study and the study group comprised of patients of all age-groups and both sexes who attended and admitted toOP/IP departments Shri B.M. Patil Medical College, Hospital and Research centre, Vijayapur and District hospital, Vijayapur. The patients with acute febrile illness, and already diagnosed as other infections like malaria, enteric fever, dengue during the sample collection were excluded from the study.

After obtaining the informed consent, 5 ml of Venus blood samples were collected from patients in plain tube and serum was separated and blood clots of all the samples were stored at -20°C.

Serological Tests

Weil Felix test: Serum samples were screened for the presence of antibodies against Scrub typhus infection by Weil felix tube agglutination test (Tulip diagnostics, Goa), in which the antibodies present in the serum reacts with antigens derived from various proteus species and exhibit agglutination. Samples with titres of 1:160and above for OXK were considered as positive for Scrub typhus infection.

IgM ELISA: All the samples were screened for the serological confirmation of the presence of specific antibodies against Scrub typhus infection by IgM ELISA (Inbios International, USA), a method which is reported to be more sensitive and specific by several researchers [3,4,8,10,11]. Both positive and negative controls provided with the kit were also included in the assay. Both the serological tests were performed strictly according to the manufacturer's instructions.

Nested PCR: Blood clot was homogenized and the DNA was extracted from 200 µl of homogenized blood using the QIAmp blood mini kit (Qiagen) as per manufacturer′s instructions. The eluted DNA was aliquoted and stored at -30ºC. Nested PCR was performed using two sets of primers to amplify a 483 base pairs segment of the gene encoding the 56 kDa antigen of *O. tsutsugamushi* as described by Furuya *et al*. (1993). The primers were procured from Integrated DNA Technologies (IDT).

Details of primers used are:

First set (Outer primer); P34: 5′-TCA AGC TTA TTG CTA GTG CAA TGT CTGC- 3′ and P55: 5′ -AGG GAT CCC TGC TGC TGT GCT TGC TGCG-3′

Second set (Inner primer); P10: 5′-GAT CAA GCT TCC TCA GCC TAC TAT AAT GCC-3′ and P11: 5′-CTA GGG ATC CCG ACA GAT GCA CTA TTA GGC-3′

Premixed ready to use green master mix (Promega, USA) was used for amplification, which consists of Taq Polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. Total volume of reaction mixture including templates was 50 µl. First PCR was performed with the template DNA using first set of primers (p34 & p55). The first PCR product was used as template for the second PCR with second set of primers (p10 & p11). Amplification protocol for both PCR was: initial denaturation of template at 95°C for 10 min, denaturation at 95°C for 30 sec,annealing at 55°C for 1min, extension at 70°Cfor 1 min for 35 cycles followed by final elongation at 70°C for 10 min in a thermal cycler (Aeris, ESCO). The amplicons were electrophoresed in a 1% agarose gel containing ethidium bromide (0.5µg/ml) and visualized in Gel documentation system system (Syngene, USA). PCR products were sequenced by Sanger sequencing method to identify the species.

Results

Out of 209 samples, 39 samples showed agglutination with OXK antigen with titre of 1:160 and above, and13 samples were positive by IgM ELISA. Interpretation was done as per the manufacturer instructions (Table 1).

Table 1: Results of comparison of two serological tests performed for the detection of Scrub typhus infection (n = 209).

Serological Tests	Total positive	IgM ELISA Positive	IgM ELISA Negative
Weil felix OXK positive	39	11	28
Weil felix OXK negative	170	02	168

Negatives include samples with titre 1:80 and below for OXK, and samples positive for other WF antigens except OXK. Samples non reactive with WF antigens were not evaluated.

In nested PCR, out of 13 ELISA positives, two samples amplified the gene encoding the 56 kDa antigen of O. *tsutsugamushi*. (Fig 1). One positive control received from Dept of Microbiology, BMCRI, Bangalore and one negative control was also tested along with the samples.

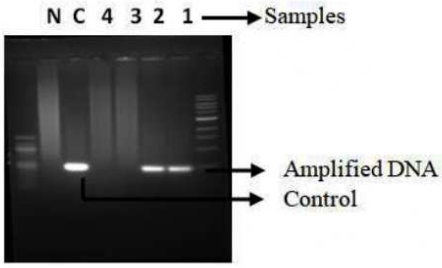

Fig 1. Agarose gel electrophoresis picture of amplified DNA by nested PCR. Lane 1- sample 1, lane 2- sample 2, lane 3 sample 3, lane 4 sample 4, lane -C positive control and lane - N negative control.

PCR product was sequenced bidirectionally using Applied Biosystems model 3730xl/ 3730XLPA-19137-017 at Eurofins genomics India Pvt. Ltd, and quality of sequences was checked with Finch TV Version 1.4 and consensus sequence was generated using Bio Edit software version 7.2. Further nucleotide sequences were queried against NCBI BLAST programme to identify the sequenced sample. NCBI BLAST program (blastn suite) showed 97 to 99% homology with the strains reported from KMC Manipal (MG283201.1), Andaman and Nicobar (MF457892.1), Pondicherry (KT970967.1) and Himalayan region (DQ286233.1), Uttar Pradesh (KR706188.1).

Discussion

Rickettsial infections are re-emerging in India and will lead to significant morbidity and mortality if failed to diagnose timely and take appropriate treatment. The diagnosis of scrub typhus is generally made by the history and clinical presentation. The vast variability and common clinical manifestations of the disease which is similar to other febrile illnesses makes the clinical diagnosis challenging. During the last decade, several researchers have reported the existence of rickettsial infection in different part of India using single or a combination of serological tests like Weil felix, ELISA and Microimmunofluorescence assay [1, 3-14] and using both Serological test and molecular test- PCR[16-19].

When we reviewed the last two years medical records of tertiary care facility Shri B.M. Patil medical college Hospital and research centre Vijayapura, we found that significant numbers of cases have been diagnosed using Weil felix test alone, and in which seropositivity for

SFG Rickettsiae or Indian Tick typhus was commonly reported than Scrub typhus. We also have observed and reported similar results in our previous serological study on R. conorii. In that study, out of 231 cases screened, 105 cases were positive for OX2 antigen by Weil felix test suggestive of R. *conorii* and 27 cases were confirmed serologically by IgM ELISA.

Though scrub typhus infection have been increasing and reported from various parts of India, Scrub typhus was not evaluated and no confirmatory data is available from north Karnataka region. Hence, we undertook this study to investigate the existence of Scrub typhus in Vijayapura area of Karnataka using both serological and molecular tests. All the cases included in the study were clinically suspected with fever of more than one week (100%), body ache (23%)and rashes (19%). We could not find eschar in any cases.

Out of 209 symptomatic cases screened, 39 (18.5%) were positive by Weil felix with titre of OXK >1:160 and 13 were positive by IgM ELISA suggestive of Scrub typhus infection. It is highly significant in this region even though the results obtained in our study shows considerable difference in percentage of positive cases reported by several researchers from different geographical parts of India like Karnataka [9,10], Andrapradesh[3, 13, 17], Pondicherry [8,16], Tamil Nadu[4,5,6,17], Himachal Pradesh[7,11,12] and Delhi [14]. We have observed more seropositivity during cooler months between August to January, and same was reported by few of the investigators [3,5,6].

Since both the serological tests exhibit significantly varied sensitivity and specificity according to the geographical area[3-13], the detection of causative organism directly from the patients sample would be highly important. Different PCR techniques have been reported during the last decade for detection of different genes of scrub typhus namely *56kDa*[15-20], *Groel* and *16SrRNA*[16] from blood samples [15-19] and rodents [20]. To substantiate the seropositivity observed in the study area, we performed the nested PCR on blood clots of ELISA positive samples for the confirmation of organism using the primers encoding the 56kDa gene of O. *tsutsugumushi* as described by *Furuya et.al.*[15]. While ELISA positive samples had high OD value, we succeeded to amplify a 483 bp segment of the gene encoding the 56 kDa antigen of *O. tsutsugamushi* in only two samples which were positive by both the serological tests Weil Felix and IgM ELISA. Results of Nucleotide sequences queried against NCBI BLAST programme showed 97 to 99% homology with the strains reported from KMC Manipal (MG283201.1), Andaman and Nicobar (MF457892.1), Pondicherry (KT970967.1), Himalayan region (DQ286233.1) and Uttar Pradesh (KR706188.1).

Conclusion

Findings of our study demonstrated that though more seropositivity of SFG Rickettsial infection is observed by Weil Felix test, scrub typhus infection is also circulating and causing acute febrile illness in and around Vijayapura, North Karnataka region. As the routinely used Weil felix test is less sensitive, inclusion of specific tests like ELISA and nested PCR is very useful in proper diagnosis and patient management.

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Conflict of Interest: None

Source of Funding: Self

Ethical Clearence: Ethical clearance was obtained from ethical committee, BLDE University Vijayapur.

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RESEARCH ARTICLE

Serological Evidence of Spotted Fever Group Rickettsial infection in North Karnataka Region

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ABSTRACT:
Background: Rickettsial diseases are important reoccurring infections that mostly go unnoticed or misdiagnosed due to low manifestation, non specific signs, symptoms and absence of widely available sensitive and specific tests. Failure of timely diagnosis leads to significant morbidity and mortality. Though the disease is rampant throughout the world, only few case reports and studies have been reported from only some states of India in the past 15-20 yrs. **Aim and Objectives:** To demonstrate the serological evidence for the existence of Spotted fever group Rickettsial infections in North Karnataka region and to develop a preliminary understanding of the distribution of these infections. **Materials and Methods:** During the period of 2015-16, a total of 231 patients sample presenting with consistent fever of more than 8 days, headache, myalgia, rashes and eschar were examined for Rickettsial infections by Weil Felix agglutination test. Further all the WF positive samples were screened for corresponding antibodies to SFG R. conorii by specific serological test IgM/ IgG ELISA. Results obtained with both the methods were tabulated. **Results:** Out of 231 cases, 105 samples showed agglutinins with Weil Felix antigens. 84 (36.36%) samples had titres ranging from 1:80 to 1:320 with OX2 antigen alone, 5 (2.16%) samples had titres of 1:80 and above for OX19 antigen and 16 (6.92%) samples had titres of 1:80 and above with both OX2 AND OX19 antigens. In R. conorii specific IgM ELISA 27 (25.7%) samples were positive with index >11 and 4 samples had equivocal index value. 74 samples were negative for R. conorii specific antibodies by ELISA. **Conclusion:** Findings of our study clearly demonstrated that among Rickettsial infections, SFG R. conorii infection seems to be common infection in and around Vijayapur. Inclusion of specific IgM ELISA method in routine diagnostic course is highly necessary for the proper treatment and patient management.

KEYWORDS: Weil Felix test, Spotted Fever Group Rickettsiosis, R. *conorii*, ELISA.

INTRODUCTION:

The Rickettsiae are small gram negative, aerobic cocco bacilli, that are obligate parasites of eukaryotic cells. The genus Rickettsia is included in the bacterial family Rickettsiaceae of the order Rickettsiales. This genus includes many species associated with human disease including those in Spotted fever group and Typhus group.

Rickettsial infections are escalating and are rampant right through the world. In India they are reported from states Maharastra, Tamil Nadu, Karnataka, Kerala, Jammu & Kashmir, Uttaranchal, Himachal Pradesh, Rajasthan, Assam and West Bengal[1,2,3,4,5].

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Rickettsial infections are one of the significant cause of fever of unknown origin, and these needs to be differentiated from other febrile illness like enteric fever, malaria, dengue etc.[6]. In view of low manifestation, non specific signs, symptoms and absence of widely available sensitive and specific tests, these infections are very difficult to identify and may pose a serious threat to public health if not diagnosed or misdiagnosed. Failure of timely diagnosis leads to significant morbidity and mortality[1,2,3].

Various serological tests are available for the diagnosis Rickettsial infections like Microimmunofluorescene, latex agglutination, ELISA, Weil felix test etc. IFA is the well recognized serological test and considered as gold standard technique, but very expensive and requires expertise[5]. As the specific serological tests are not easily available and expensive, Weil felix test is routinely used in most of the laboratories though the sensitivity and the specificity of the test is very low.

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Since there was no complete study on Rickettsial infections in North Karnataka, this study was undertaken during the period 2015 - 2016 to know the serological evidence for the existence of Spotted fever group Rickettsial infections and to develop a preliminary understanding of the distribution of these infections.

MATERIALS AND METHODS:

The study group comprised of patients of all age-groups and both sexes attended and admitted to OP/IP departments of Medicine, Pediatrics and Dermatology of Shri B.M. Patil Medical College, Hospital and Research centre, Vijayapur and District hospital, Vijayapur (in and around Vijayapur) presenting with consistent fever of more than 8 days, headache and myalgia, rashes and eschar. The patients with cause of fever already known during the sample collection were excluded from the study. As there was no prevalence study on SFG Rickettsiae in north Karnataka region, we have considered the proportion of symptomatic cases tested for Rickettsial infections during the year 2010- 2011 for calculating the sample size. Calculation was done by using the formula $n= (Z)^2 \times p \times (1-p)/l^2$ where z= confidence level- 95%, p= proportion – 60%, I = marginal error – 4%. Ethical clearance certificate was obtained from ethical committee, BLDE University Vijayapur.

After obtaining the informed consent, 5 ml of blood was collected from the patients in plain tube and serum was separated. All the serum samples were tested for the detection of Rickettsial antibodies by Weil felix test, it exhibit agglutinins to Proteus vulgaris strain OX 19, OX 2 and OX K. (Tulip diagnostics, Goa). Samples with titres of 1:80 and above for OX2 and OX19 were considered as positive. For the serological confirmation of R. conorii infection, all the Weil felix positive samples were screened for Rickettsia conorii by IgM/IgG ELISA (Vircell, Granada, Spain), a specific serological test wherein antibodies present in the serum reacts with SFG R. conorii specific antigen Moroccan strain (ATCC VR-141) coated in the plate. Positive and negative controls were provided by SF ELISA kit manufacturers, Vircell Microbiologica, Spain. Test was performed strictly according to the manufacturer's instructions.

The interpretation of the result was made as follows: Antibody index <9 were considered negative, 9–11 were equivocal (needed repeat testing) and >11 were considered as positive for specific antibodies against R. *conorii*/ SFG Rickettsiosis.

RESULTS:

Weil Felix tube agglutination test:

All the serum samples were tested by Weil Felix tube agglutination test with different sample dilutions for the detection of anti –Rickettsial antibodies. Agglutination observed in the dilutions ranging from 1:80 to 1:320 with OX2 antigen, OX19 antigen and both O2 & OX19 antigens were considered as positives.

The results are reported in (Table 1).

Table 1: Year wise details of the patients tested for R. infections by Weil Felix test and ELISA

Sl. No	Year	n	Weil Felix test results			
			OX 2	OX 19	OX 2 & OX19	Negative
1	2015	109	38 (34.8%)	2 (1.83%)	9 (8.25%)	60 (55.04%)
2	2016	122	46 (37.7%)	3 (2.45%)	7 (5.73%)	66 (54.98%)

n = Total number of samples subjected in our study

R. conorii IgM ELISA:

All the Weil Felix positive samples were subjected to more specific immunological test Rickettsia conorii IgM ELISA for the confirmation of presence of anti – Rickettsial antibodies in the serum. OD values of all the samples were recorded and interpreted. The results in comparison with Weil Felix test are reported in (Table 2).

Table 2: Results of both WF test (antigens wise) and R. conorii IgM ELISA.

Sl. No	WF test - Antigens	WF Test Positives	IgM ELISA Positives
1	OX 2	84	24
2	OX 19	5	0
3	OX 2 & OX 19	16	3
Total		**105**	**27**

Gender wise analysis of the results obtained in the study is recorded in the (Table 3).

Table 3: Gender wise distribution of Weil Felix and IgM ELISA test results.

Tests	Male (128)	Female (103)
Weil Felix (n-231)	59(46.09%)	46 (44.66%)
IgM ELISA (n-105)	14 (23.72%)	13 (28.26%)

n- Samples subjected for test.

DISCUSSION:

Present work was carried out to serologically confirm the occurrence of SFG Rickettsial infection in and around Vijayapur, Karnataka by well established WF test and more specific serological test SFG R. *conorii* specific IgM ELISA test. We found significant number of cases positive by WF test. Out of 231 cases screened for R. infections 105 (45.45%) were positive for OX2 & OX19 suggestive of SFG Rickettsial infection. The results obtained in our study shows considerable disparity with similar studies reported by other investigators. In a study from Delhi by Veena Mittal *et.al* had reported 8 (27.5%)

SFG rickettsiae positive cases out of 29 cases positive by WF test[6]. Kumar *et.al* from Karnataka had reported seroprevalence of 23.3% in and around Davanagere[8].

For the serological confirmation, we screened all WF positive samples further by another serological test R. *conorii* IgM ELISA. IgM ELISA proved to be highly sensitive and specific in some of the studies carried out by other investigators during last 10 years. According to the manufacturers of the kit, the sensitivity and specificity of Vircell R. *conorii* IgM ELISA kits are 94 and 95%; and for IgG ELISA kits, these are 85, 100%, respectively[15]. Do *et al*, in their study observed that the sensitivity for R. *conorii* Vircell IgM + IgG ELISA was 90% and specificity was 100%[10]. In the present study out of 105 WF positive cases 27 (25.7%) cases found positive by R. *conorii* IgM ELISA. Though the present study clearly demonstrates the seroprevalence of R. *conorii*/ Indian tick typhus infection in this region, the figures obtained in our study notably vary from some of the studies reported by other investigators in India. Kalal *et al*, from Karnataka reported that 37.1% hospitalized children were seropositive for SFG rickettsia based on the IgG ELISA result in acute samples and some paired sera[9]. Somashekar *et al* had reported seroprevalence of SF in children of Tamil Nadu was found to be 7.78% during 2003–2004[3]. Tripathi *et al* from Uttar Pradesh reported seroprevalence of spotted fever in 42.15% in children and 17.10% adults. Their observation was based on IgM ELISA and IgM IFA positivity on acute samples only[11]. In a study by Stephen *et al* demonstrated a high seroprevalence of SFG rickettsiosis (44.38%) in Puducherry region of south India. A moderately higher rate of ST and SFG coinfection/cross-reactivity (34.50%) was also observed in the SF IgG positive patients[7]. In the present study more seropositivity was observed during cooler months between August to December, same was reported by most of the investigators in their study[2,12,13,14].

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CONFLICT OF INTEREST:

None.

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