DISCUSSION
CHAPTER V

DISCUSSION

5. Discussion:

5.1. Seroprevalence of Scrub typhus among febrile ill patients: a preliminary study

Rickettsial infections are re-emerging and lead to significant morbidity and mortality if failed to diagnose at appropriate time (Rathi et al., 2010). In India the burden of rickettsiosis is underestimated as there is lack of both community based studies and availability of specific laboratory tests (Chugh, 2008). The presence of rickettsiosis has been documented from Jammu and Kashmir, Himachal Pradesh, Uttarakhand, Rajasthan, Assam, West Bengal, Maharashtra, Kerala and Tamil Nadu (Mathai et al., 2001; Sundhindra et al., 2004; Mahajan et al., 2006; Batra, 2007) but there are no reports from Andhra Pradesh. There are also no reports from other states in India like Vidharba, Madhya Pradesh and Chhattisgarh, but the factors predisposing to rickettsial infections are prevalent in this part (Rathi et al., 2011). Rickettsiosis constitute a very significant, but often unrecognized portion of the acute febrile disease contributing to financial burden on many populations, especially in developing countries (WHO, 1993). These zoonotic infections should be considered in differential diagnosis of any acute febrile illness. Rickettsiosis should be differentiated from other infections like meningococcemia, brucellosis, malaria, viral illness and typhoid fever as there is an overlap of clinical features (Shah et al., 2009). A high index of suspicion is required to diagnose rickettsiosis especially in endemic areas. Due to lack of simple diagnostic tools, the diagnosis of scrub typhus cannot be easily made in many laboratories. For diagnosis of rickettsiosis, gold standard diagnostic tests like indirect immunofluorescence antibody (IFA) and indirect Immunoperoxidase (IIP) requires highly trained persons to perform and production of antigens may vary among different laboratories leading to inconsistency in interpretation of results.
In several regions around the world, Weil-Felix test has been used in documenting the presence of rickettsial infections for the first time (Parola et al., 2005). Usually Weil-Felix results may be negative during the early stages of the disease because agglutinating antibodies are detectable only during the second week of illness (Amano et al., 1992). Isaac et al., have demonstrated that the sensitivity of Weil-Felix test is 30% at a break point titer of 1:80, but the specificity and positive predictive value were 100% (Isaac et al., 2004). In another study by Prakash et al., Weil-Felix test showed specificity of over 98% and a sensitivity of about 43% (Prakash et al., 2006). Instead of misdiagnosing rickettsial infections Weil-Felix test can be used to detect more positive cases (Suzuki et al., 1980). By performing Weil-Felix simply we can get some information regarding the nature of infection, which can be cross confirmed by other techniques, if available. There is good correlation between the results of Weil-Felix test and detection of IgM antibodies by IFA/ELISA (La Scola et al., 1997).

In this study we used single acute phase sera from patients with pyrexia of unknown origin attending the hospital for determining antibodies against SFG, TG and ST. The prevalence of antibodies to scrub typhus was highest 79 (55.63%) followed by typhus fever group 28 (19.71%) and spotted fever group 21 (14.78%) respectively. A study conducted by Mittal et al. on fever of unknown origin patients sera showed that 42.6% were positive for OXK, 39.3% were positive for OX2 and 8.1% were positive for OX19 (Mittal et al., 2012). In another study conducted by Kamarasu et al. showed 9.2 % of patients sera were positive for scrub typhus and 4.6% for other rickettsiosis (Kamarasu et al., 2007). There results show that scrub typhus seems to be more common when compared to other rickettsiosis. Kulkarni et al., from Western part of India reported higher incidence of spotted fever group (Kulkarni et al., 2009). Rathi et al., also reported that of the 75 patients with rickettsial infections, 52 (69.3%) had spotted fever and 23 (30.7%) scrub typhus. Our study showed more positives for scrub typhus followed by typhus fever and spotted fever group. In the present study more number of scrub typhus cases was observed from October to February. In southern India outbreak of scrub typhus occur during cooler months as reported by Mathai et al. (Mathai et al., 2003). One of the drawback our study is that we could not be analyze paired sera i.e. acute and convalescent phase serum samples.
For the present study only single Weil-Felix test was used to diagnose whether ricketsiosis occurs in this region are not. Weil-Felix test is the oldest assay based on detection of antibody to various *proteus* antigens that cross react with rickettsiae. By using this simple, economical Weil-Felix test we can guide a clinician in instituting appropriate treatment particularly in areas where rickettsiosis have not been previously diagnosed. Whole cells of *P. vulgaris* OX2 react strongly with serum from person infected with spotted fever group (SFG) rickettsiae with the exception of those with Rocky mountain spotted fever (RMSF); and whole cells of *P. vulgaris* OX19 react with serum from person infected with typhus group rickettsial as well as with RMSF. Also, OXK strain of *P. mirabilis* agglutinates with serum from scrub typhus patients (Amano *et al*., 1992) Gurung *et al*., reported more scrub typhus cases were positive by ELISA and ICT tests than Weil-Felix test (Gurung *et al*., 2013). Although Indirect IgM ELSA may give false positive results due to rheumatoid factor (Jang *et al*., 2003) and false negative results due to rise of IgG levels occur at the time of secondary infection (Kelly *et al*., 2009). Primary infection produces a rapid rise in IgM antibodies within 8 days, whereas secondary or re-infection is characterized by a sharp rise in IgG levels, with a variable IgM response (Kelly *et al*., 2009).

5.2. Comparison of three diagnostic modalities – Weil-Felix test, Enzyme Linked Immunosorbent assay and Nested PCR for detection in blood.

Scrub typhus is a common disease in Asia (Elisberg *et al*., 1967; Brown *et al*., 1976; Brown *et al*., 1984; Tamura *et al*., 1984; Richards *et al*., 1997) in both indigenous and visiting individuals (Philip, 1948; Berman *et al*., 1973; Olson *et al*., 1977; Watt *et al*., 1994; Eamsila *et al*., 1996; Gormley, 1996: Richards *et al*., 1997; Kelly *et al*., 2002). It is only recently recognized as an underestimated but important cause of acute undifferentiated fever in South-East Asia and the Pacific regions (Suttinont *et al*., 2006). Recent reports of antibiotic breakthroughs or delayed treatment because of early misdiagnosis have raised concerns among medical professionals. The misdiagnosis often occur in nonindigenous populations or outside regions where scrub typhus is historically endemic, possible because of increases in ecotourism in what are now relatively
politically stable regions (Kelly et al., 2009). In India Scrub typhus was recognized as a typhus-like fever in 1917 and in subsequent outbreaks (Tattersall, 1945). It was a major cause of fever among troops during World War II in Assam-India-Burma border (Sayen et al., 1946). There was a resurgence of the disease in 1990 in a unit of an army deployed at the Pakistan border of India (Singh, 2004). Scrub typhus has been reemerging in the Indian subcontinent (Mathai et al., 2003; Lewis et al., 2003; Kularantne et al., 2003). It has been reported from neighboring states (Jammu & Kashmir, Andhra Pradesh Kerala, Tamil Nadu, Pondicherry, West Bengal, Kerala, Tamil Nadu, Pondicherry, Himachal Pradesh, Nagaland, Rajasthan, Haryana Maharashtra, Karnataka, Sikkim, and Uttarakhand) but there is paucity of data on this problem from Andhra Pradesh. Although most studies from Tamil Nadu are from one institute (Mathai et al., 2001), documentation has been done in at least 15 districts (Kamarasu et al., 2007). The reported number of cases of scrub typhus from different parts of the country particularly from large tertiary care hospitals do not give a true picture of prevalence of scrub typhus in the country (Chogle, 2010).

Transmission of disease occurs throughout the year in the tropical areas, whereas in the temperate zones transmission is seasonal (Chang, 1995). Seasonality of the disease is determined by the appearance of larvae, is observed mainly in the autumn but also in spring (Raoult, 2009). In the present study most of the cases were observed during the months of July to November. Such post monsoon outburst is reported earlier also (Sharma et al., 2005; Varghese et al., 2006; Somashekar et al., 2006; Kamarasu et al., 2007; Vivekanandan et al., 2010; Narvencar et al., 2012), because during the months of August to October, farmers are involved in the harvesting activity in the fields, where they are exposed to the bites of larval mites (Narvencar et al., 2012). And also in the immediate post monsoon period (September to early months of the next year), there is growth of secondary scrub vegetation, which is the habitat for trombiculid mites (mite islands) (Tilak, 2009). Scrub typhus is found throughout the India and is reported seasonally from August through October, with L. deliense being the primary vector (Kalra, 1952). During World War II, cases were reported throughout the year in the Assam-India-Burma border region but primarily from October through December, and
the mortality rate was 5% (Tattersall, 1945; Sayen et al., 1946). Mathai et al., reported an outbreak in Tamil Nadu state in southern India that occurred from October 2001 through February 2002 (Mathai et al., 2003). A study from Sikkim and Darjeeling also reported seasonal phenomenon of the disease (Sharma et al., 2009; Gurung et al., 2013).

In the present study, most patients were males compared to females. In a study by Sharma et al. reported more females were affected than males because they more commonly worked in the fields (Sharma et al., 2005). A study from Rajastan reported that positivity for scrub typhus was significantly higher among female who were suffering from fever of unkown origin in comparision to males (Bithu et al., 2014). In the present study it was observed that most of the patients were farmers. Watt et al. reported that in Thailand, leptospirosis and scrub typhus were associated with rice farmers, an occupational group known to be at high risk (Watt et al., 2003). Most of the patients studied in our study belonged to rural area than urban area. The people living in urban area are traditionally considered to be at low risk (Wang et al., 2013).

Scrub typhus presents as an acute febrile illness with non-specific signs and symptoms (Mahajan, 2005). It accounts for up to 23% of all febrile episodes in areas of the Asia-Pacific region where scrub typhus is endemic and can cause up to 35% mortality if left untreated (Brown et al., 1976). In the past, the clinical diagnosis of scrub typhus was dependent on detecting eschar and rash and on the history of outdoor activity (Blake et al., 1945(a); Sayen et al., 1946; Berman et al., 1973). However differentiation of scrub typhus from other acute tropical febrile illness, such as dengue fever, leptospirosis, malaria, viral hemorrhagic fevers, is difficult to because their signs and symptoms are very similar (Chen, 2001; Chierakul et al., 2004). Presence of eschar was also shown to be important finding for diagnosis of rickettsial pox, cutaneous anthrax, tick-borne rickettsiosis and other diseases, and travel and other population migrations are currently often occurring (Chogle, 2010). Although eschars have high diagnostic value, the lesions are painless and without any itching sensation in most cases, causing the infection to be undetected by most patients. In addition, an eschar is similar to a scab formed after trauma, and its size may be very small, which also hinders detection of eschar in many cases (Chayakul et al., 1988; Tsay et al., 1998).
An eschar is usually seen less on Caucasian and East Asian patients but is seen less frequently on South Asians, especially those who are dark skinned (WHO, 1974). The symptomatic diagnosis of scrub typhus is sometimes difficult because the skin manifestation resembles a drug sensitivity reaction (Manosroi et al., 2006). The presence of eschar is highly suggestive of scrub typhus but is reported to occur in a variable proportion of patients (from 7% to 97%) (Ogawa et al., 1998; Silpapojakil et al., 2004). This definition is not suitable for the Indian subcontinent since eschar and rash were seen in less than 10% of cases. Scarcity of primary lesion has been noted in earlier reports from India this may be due to variation in strain types in this area (Varghese et al., 2006).

Eschar and skin rash resulting due to vasculitis are the major clinical findings for diagnosing scrub typhus in our study we have observed only 4.42% of patients are presenting with eschar whereas only 8.84% patients with eschar. Orientia infects vascular endothelium leading to vasculitis and organ dysfunction (Kothari et al., 2006). In the present study presence of eschar is reported as 4.42%. It is difficult to search for evidence of an eschar unless a thorough examination of the body. The distribution of eschar on body surface might be associated with dressing styles and personal hygiene, as the two factors affect how and where chiggers entered and stayed on the body surface (Wu, 2000; Chen, 2000; Chen, 2001). However, individuals living in rural areas are often reluctant to accept a through body examination because of cultural or other reasons (Chen 2001; Chierakul et al., 2004). Acute undifferentiated fever was the common feature in all the patients. Rash is usually observed at the end of the first week in half of primary infections, being recognized in a smaller proportion of those reinfected and with dark skin. A study in India by Singh et al. reported that eschar was not seen in any patient (Singh et al., 2014).

In our study, most common feature was fever with cough (34.51%). Gastrointestinal signs and symptoms such as vomiting and abdominal pain was also seen in most of the patients. A study by Kedareshwar et al., shown predominance of gastrointestinal signs and symptoms such as nausea and vomiting, abdominal pain, and loose motions (Narvencar et al., 2012). Elevated transaminase levels had the best predictive values in identifying scrub typhus in patients with undifferentiated febrile
illness. In this study elevated transaminases and thrombocytopenia was seen in only 5 patients. However these findings were analyzed in only few cases and possible to perform in all the cases. Multiorgan dysfunction was seen in only 2 patients who were not received tetracyclines or macrolides as they are admitted late. In most of the cases tetracycline was effective with a majority of patients showing dramatic defervescence within 24 h (Blacksell et al., 2007).

Isolating *O. tsutsugamushi* requires biosafety level-3 facilities and culture on cell monolayers; median time to positivity is 27 days (Luksameetanasan et al., 2007). Mouse inoculation is even more laborious and intensive on resources (Casleton et al., 1998). The oldest test in current use is the Weil-Felix OX-K agglutination reaction, which is inexpensive, easy to perform, and results are available overnight; however, it lacks specificity and sensitivity (Kelly et al., 1988). For initial diagnosis of scrub typhus in the present study Weil-Felix test was used. This test showed more positives when compared with ELISA and N-PCR tests. It was also seen that there was good agreement between Weil-Felix test and ELISA when compared with N-PCR. So Weil-Felix test and ELISA tests can be used in laboratories where PCR is not available. PCR methods when used independently or in conjugation with Weil-Felix test can be employed as a specific diagnostic tool for diagnosis of scrub typhus in developing countries and aid in the surveillance and effective treatment of this emerging infectious disease (Bakshi et al., 2007). However, a larger number of unknown febrile sera should be used for further evaluation of this test compared with other serological tests.

Previously serological assays which include the indirect fluorescence assay (IFA), indirect immunoperoxidase assay, enzyme-linked immunosorbent assay (ELISA), and dot blot assays, use rickettsiae grown in host cells or extracts of purified bacteria as antigens (Bozeman et al., 1963; Dohany et al., 1978; Dasch et al., 1979; Suto, 1980; Yamamoto et al., 1982; Kelly et al., 1988; Weddle et al., 1995; Suwanabun et al., 1997). ELISA appears more suitable than the IFA for rapid screening of large numbers of sera in epidemiological surveys because objective measurements of titer may be obtained with a single serum dilution, provided the titer of the serum is not exceptionally high (Dasch et al., 1979). In the present study IgM ELISA was used for testing the clinically suspected
scrub typhus samples. It was found that ELISA positive samples were also positive by N-PCR.

Bourgeois et al., found that two types IgM responses occurred in scrub typhus patients in areas of endemicity by MIF. Type 1 responses, which were believed to be primary infections, exhibited an early, greater, and more rapid increase in IgM responses compared to IgG responses, IgM responses were relatively more strain specific. Type 2 responses had suppressed and delayed IgM responses which were highly strain specific, while their IgG responses were immediate, strong, and not strain specific (Bourgeois et al., 1982). A commercially available ELISA for immunoglobulin M (IgM) and IgG detection using r56 has been developed and evaluated previously (Land et al., 2000). The r56 IgM assay may be even more sensitive to differences in immune responses to the infecting strains than the IIP or the MIF assay, because no other conserved antigens are present as found in whole organism assays (Ching et al., 1998). The ELISA format is very convenient for large scale testing in laboratory and takes about 50 min to perform (Ching et al., 2001). The serological tests have low sensitivities in the early stage of scrub typhus due to insufficient production of antibodies, frequent follow-up tests are needed (Bozeman et al., 1963). Detection of the organism or its DNA seems more logical to antibody detection because it indicates active disease and also valid when used in the endemic area of infection, where the background antibodies often interfere with the interpretation of antibody assay (Bakshi et al., 2007).

The scrub typhus ELISA is a flexible alternative to the IFA technique (Dasch et al., 1979) and have specificities and sensitivities of >90% for detecting specific antibodies (Varghese et al., 2006). In moderately equipped laboratories in endemic regions, recombinant antigen-based ELISA is a useful alternative technique for diagnosis (Land et al., 2000). The r56 protein is an immunodominant outer membrane antigen which contains conserved domains that may account for the cross reactivity of antisera against diverse serotypes, while the variable domains are likely responsible for some less cross reactivity with other rickettsial groups (Kim et al., 1993; Ching et al., 1998; Choi et al., 1999).
Scrub typhus runs a mild clinical course and responds well to proper antibiotic therapy. However in patients with a delayed diagnosis, it may cause fatal complications (Silpapojakul et al., 1991). An accurate diagnosis of scrub typhus often relies on laboratory tests such as mouse inoculation (Carley et al., 1955) or other serological methods (Brown et al., 1983; Pradutkanchana et al., 1997). Since the mouse inoculation technique is hazardous, complex and does not yield results in time to influence patient management, the standard diagnostic test for scrub typhus relies mainly on demonstration of serological response against *O. tsutsugamushi* by either immunofluorescent antibody (IFA) (Brown et al., 1983) or indirect immunoperoxidase (IIP) (Pradutkanchana et al., 1997) assays. Serological tests are limited by the presence of high background titers in endemic populations (Blacksell et al., 2007), low sensitivity when antibody titers are still too low to be detected and a fatal seronegative case of scrub typhus has been reported when testing admission samples (Sugita et al., 1997). Although *Orientia* infection can be identified by serological tests, patient sera often cross-react with the antigens from different strains and it is unclear whether the cross-reaction is due to strains that contain a mosaic of antigenic determinants (Shirai et al., 1981, 1982(a); Kelly et al., 1988). Serologic tests have low sensitivities in the early stage of scrub typhus due to insufficient production of antibodies, frequent and also frequent follow-up tests are needed (Bozeman et al., 1963). Thus, a rapid early and accurate diagnosis of scrub typhus is essential for specific and effective treatment.

PCR assays have been widely used for rapid identification of fastidious organisms or rickettsiae that are difficult to cultivate (Furuya et al., 1991, Murai et al., 1992; Furuya et al., 1993; Sugita et al., 1993; Shieh et al., 1995) and also possible to detect *Orientia* at the onset of illness when antibody titers are not high enough to be detected (Kelly et al., 1990). The conventional PCR requires 20 ng of DNA for detection whereas Nested PCR requires only 200 pg of DNA, thus N-PCR is 100 times more sensitive than conventional PCR (Murai et al., 1992; Kim et al., 2011). Nested PCR targeting 56-kDa major outer membrane protein antigen of *Orientia* is known to be 100 times more sensitive than performing single PCR for detecting *Orientia* DNA (Murai et al., 1992). It has been reported that nested PCR enabled the detection of five copies of *O. tsutsugamushi* DNA
in a specimen (Kim et al., 2006). Blood clot was used for DNA extraction which is usually discarded after serum separation. The quality of DNA was good when we checked it by Nanodrop (Thermo Scientific, 8000). PCR amplification of the 56 kDa protein gene has been demonstrated to be a reliable diagnostic method for scrub typhus it elicits very early IgM and IgG serologic responses (Murai et al., 1992). Kim et al compared C-PCR and N-PCR targeting TSA gene for diagnosing scrub typhus and reported that N-PCR had a sensitivity of 87.8% and specificity of 100% (Kim et al., 2011). Highly sensitive PCR methods have made it possible to detect Orientia at the onset of illness when antibody titers are not high enough to be detected (Kelly et al., 1990; Furuya et al., 1991). Saisongkorh et al. reported that DNA of the organism will persist in blood for as long as 22 days after onset of disease in patients without specific treatment and 27 days after therapy (Saisongkorh et al., 2004).

The major polypeptides of Orientia are proteins of 70, 60, 56, 47, and 25 kDa (Tamura et al., 1985). The 56 kDa protein of Orientia comprises 10-15% of the total rickettsial cellular protein content that may be involved in penetration into host cells (Hanson, 1985; Ohashi et al., 1989), it can be recognized by sera of 95-99% of patients with scrub typhus (Eisemann et al., 1981; Ohashi et al., 1988; Kim et al., 1993). It is an immunodominant antigen and the well-known antigenic differences that exist among various strains of Orientia depend largely on variations in the 56-kDa antigen (Tamura et al., 1985; Hanson, 1985; Murata et al., 1986; Ohashi et al., 1989). The 56-kDa is unique to O. tsutsugamushi plays a role in the adhesion and internalization of Orientia into host cells, and antibodies against this antigen can block Orientia infection of fibroblasts (Hanson, 1985; Seong et al., 1997). The 56-kDa TSA gene has an open reading frame (ORF) of approximately 1600 base pairs (bp) encoding a protein containing between 516-541 amino acid residues and consisting of 4 hypervariable regions, namely VDI, VDII, VDIII, and VDIV (Stover et al., 1990a; Ohashi et al., 1992). These 4 variable domains provide the most useful data for genetic differentiation studies within the Orientia genes (Kelly et al., 2009). TSA reacts strongly with homologues antiserum but faintly or moderately with heterologous antisera, indicating that it contains both group-specific and
type-specific epitopes which are useful for serotyping and strain classification (Tamura et al., 1985; Blacksell et al., 2008)

*O. tsutsugamushi* is characterized by a very high genomic plasticity, due to gene duplication, rearrangements, repeats, transposons and conjugative elements (Cho et al., 2007; Nakayama et al., 2008). Plasticity is increased by the presence of foreign sequences acquired through horizontal transfer (Nakayama et al., 2008). The occurrence of extensive recombination playing a key role in genetic diversity (Sonthayanon et al., 2010) along with duplication and rearrangement.

The overall plasticity is likely to be involved in host-driven selection, the capacity for adaptation to a novel environment (i.e. host) and the capacity for evading host-defenses (Cho et al., 2007; Nakayama et al., 2008). Due to extensive plasticity, the 56-kDa TSA gene represents a good target to investigate population structures. In addition to its immunological properties (Hanson, 1985; Ohashi et al., 1989), it is involved in cell invasion through specific binding to fibronectin (Lee et al., 2008). TSA interacts directly with the mammalian host, and is therefore subjected to host-driven adaptive selection (Duong et al., 2011).

More recently, molecular methods including PCR amplification, RFLP markers and genetic sequencing of specific products, target the 56-kDa type specific antigen (TSA) (Ohashi et al., 1990; Stover et al., 1990a, b; Furuya et al., 1991; Kelly et al., 1994). Different genotypes associated with different *Orientia* serotypes can be identified by analysis of variable regions of this gene without isolation of the organism (Furuya et al., 1991; Kelly et al., 1994; Horinouchi et al., 1996; Ohashi et al., 1996; Tamura et al., 1997; Enatsu et al., 1999). The 56-kDa protein is reactive with group-specific and strain-specific monoclonal antibodies, suggesting that the existence of both group-specific and type-specific epitopes (Ohashi et al., 1992). However gene amplification requires sophisticated instrumentation and reagents generally not available in most rural medical facilities. Despite of growing knowledge on epidemiology and phylogeny of *Orientia* strains in endemic regions, there is a true paucity of information from India. Despite of growing knowledge on epidemiology and phylogeny of *Orientia* strains in endemic regions, there is a true paucity of information from India (Kelly et al., 2009). For
genotyping we have targeted the TSA gene as it is a major outer membrane protein containing both group-specific and type-specific epitopes, which are useful for diagnosis of scrub typhus (Ohashi et al., 1990). The genotyping method also has the advantage of detecting unknown serotypes that are not identified by assays that are based on reactions with monoclonal antibodies against known strains (Park et al., 2010). By using PCR we can diagnose the disease at initial stage, so that we can delay or abolish antibody response by starting diagnosis at initial stage (Smadel, 1954). In addition, as antigens are unspecific, cross reaction have been described, mostly in sera of patients who have already been infected by both proteus and other α-Proteobacteria with similar antigen epitopes, such as Legionella spp. and Brucella spp.

In our study, we found that our study strains were similar to JG type that refers to ‘Gilliam type in Japan” on the basis of serological cross-reactions (Tamura et al., 2001). But by the analysis of sequences by sequence similarity JG is closely related to Kawasaki but not to Gilliam and forms significant group within Orientia species. Kawasaki type sequences have been found in Japan and China, where as JG and JG-v sequences have been found in Japan, China, and South-east Asia (Kelly et al., 2009). A study from Himachal Pradesh, India reported that their study strain sequences both ISS-1 and ISS-2 and closely matched the Japanese Gilliam cluster (Bakshi et al., 2007). In another study from India, among the two new genotypes that were causing scrub typhus in Himalayan regions one was between karp and JP-1 where as other was between Saitama and JG type (Mahajan et al., 2006). Karp like and kato like strains have been reported in Vellore, Tamil Nadu (Varghese et al., 2013). Various genotypes including the Boryong, Kato, Neimeng-65, Kawasaki, and Gilliam strains are reported from Korea (Chang et al., 1990). A recent phylogenetic analysis of Orientia from Taiwan reported two unique sequence types along with with Karp-related, Saitama, JG-related, and Kuroki-related genotypes (Lu et al., 2010). In Australia and Taiwan a very high incidence of the TA716 strain has been reported (Yang et al., 2012; Shirai 1982). Seasonal strain variations have been demonstrated in Chnese and Taiwanese studies (Liu et al., 2009; Lu et al., 2010). It can be said that there exists genotypic variation among the strains prevalent in India which needs further evaluation.
5.3. Molecular detection of clinically relevant *O. tsutsugamushi* serotypes from Southern Andhra Pradesh, India.

In this present study, we have presented the first report on circulating serotypes of *O. tsutsugamushi* in Southern Andhra Pradesh region. The public health importance of *O. tsutsugamushi* with the geographical differences in pathogenicity (Kawamura *et al*., 1995) and the emergence of antibiotic-resistant strains (Watt *et al*., 1996) have stimulated numerous studies on this organism. *O. tsutsugamushi* has over 20 antigenically distinct, regionally distributed serotypes (Kelly *et al*., 2009). Conventional serotyping of *O.tsutsugamushi* is limited to reference laboratories because of the complexity of the procedure and bio-safety considerations (Park *et al*., 2010). This is one of the reasons that the prevalence data from endemic regions are sporadic (Kelly *et al*., 2009) and the clinicians are not easily engaged in the clinical studies relate to the serotypes or genotypes of *Orientia*. The pathogenicity of *O. tsutsugamushi* for humans varies depending on geographic location, because of differences in serotypes of *Orientia* (Kawamori *et al*., 1992; Kawamura *et al*., 1995) and the disease severity and manifestations vary widely from asymptomatic to fatal and show marked geographical differences. The general course of the disease and the prognosis vary considerably depending on the character of the endemic strain (Ebisawa, 1995). Originally, the antigenic diversity of the three prototype strains, Gilliam, Karp and Kawasaki were illustrated (Shishido *et al*., 1962). Later on additional antigenic types were described, with representative strains including the Kawasaki type (Yamamoto *et al*., 1986), Kuroki type (Ohashi *et al*., 1990), Shimokoshi type (Tamura *et al*., 1984) and other distinct serotypes being present in the tsutsugamushi triangle (Ohashi *et al*., 1996). Antigenic heterogeneity of the organism may be the reason for frequent outbreak and reinfection. It is important to identify geographical distribution of existing serotypes of *Orientia* as the virulence of *Orientia* varies based on the different serotypes. Based on virulence Gilliam, Karp, Kato are considered as high virulence group whereas Boryong, Kuroki, Kawasaki and Shimokoshi were considered as low-virulence group. The virulence of *Orientia* differed depending on genetic differences between mouse strains (Groves and Osterman, 1978). Nagano *et al*., classified *O. tsutsugamushi* into three groups: a highly virulent
group including the Karp, Kato and KN-3 serotypes, a low virulence group including the Kuroki, Kawasaki, and KN-2 serotypes, and an intermediate virulence group including the Gilliam serotype. They also reported that although there may be differences in the virulence of Orientia in mice and humans, deaths due to Kawasaki and Kuroki infection are rare, and most deaths in the Northern part of Japan have been due to genotypes other than Kawasaki and Kuroki (virulence in mice of Orientia isolated from patients in a new endemic area of Japan). Determining the Orientia serotypes in endemic areas is important for accurate serologic diagnosis, the development of vaccines in future, clinical virulence and definitive diagnosis of scrub typhus (Park et al., 2010; Lee et al., 2011). A correspondence between rickettsial serotype and the species of vector chiggers has been reported, such as the transmission of the Kato serotype by Leptotrombidium akamushi, of Japanese Karp and Gilliam by Leptotrombidium pallidum and of Kawasaki by Leptotrombidium scutellare (Kawamori et al., 1992; Ogawa et al., 2008).

Moreover there is significant antigenic and genotypic diversity of this organism from the endemic areas. The diagnosis of scrub typhus is often missed due to non-specific signs and symptoms and non-availability of the relevant laboratory tests. Kim et al., reported that they found a significant differences in frequencies of eschars, rashes, general weakness, and conjunctival injection between Boryoung and Karp cluster (Kim et al., 2011). They also suggested that frequency of eschars and rashes in scrub typhus patients may depend on the genotypes of O. tsutsugamushi but they studied a very low number of Karp cluster. However, in our study we did not find any significant difference in clinical features between the Karp and Kawasaki serotypes found in this area. The presence of the pathogenomic eschar is an important diagnostic clue for diagnosis of scrub typhus (Sayen et al., 1946), but this definition is not suitable for the Indian subcontinent as eschar and rash are seen in less than 10% of cases (Varghese et al., 2006). There is less accessibility to the gold standard test of indirect immunofluorescence antibody (IFA) or indirect immuneperoxidase (IIP) because of the non-availability of the epidemic or endemic serotypes of whole O. tsutsugamushi bacteria from the particular geographic region added to the cost effectiveness of these tests (Kim et al., 2013).
Testing of different serotypes (namely Gilliam, Karp, Kato and Kawasaki) is necessary since these strains are antigenically distinct (Ching et al., 2001). Out of the 71 N-PCR positive patients samples, 67 (94.36%) were amplified by Karp specific primers and Kawasaki specific primers and the other 4 samples were not amplified by other sets of specific primers. This may be due to the absence of Orientia specific DNA in the blood samples due to institution of specific treatment before collecting the samples, while the IgM was still present in the blood. Another reason may be the non-availability of the primers needed to identify the diverse serotypes of this organism which are present in nature. The 56-kDa protein gene and 16s rRNA gene has been used for differentiating between Orientia and other genera/species (Enatsu et al., 1999). The 56-kDa gene is more useful for differentiating between O. tsutsugamushi strains than 16s rRNA, because the 16s rRNA genes of the Gilliam, Karp, Kawasaki and Kuroki strains show ≥ 98.4% homology (Ohashi et al., 1995). We targeted 56-kDa type-specific antigen gene as it contains four variable domains (Variable domains I to IV) that differ between the strains (Ohashi et al., 1992). It has been reported that O. tsutsugamushi has a variety of serotypes, the prevalence of which vary between different endemic areas (Traub et al., 1974; Shirai et al., 1982). In our study we found that Karp serotype was predominant followed by Kawasaki. Other serotypes namely Gilliam, Kato and Kuroki were not detected in our study. It was reported that Kawasaki and Kuroki type rickettsiae are transmitted by L. scutellare whereas Gilliam and Karp types by L. pallidum (Ohashi et al., 1992). A study from Himachal Pradesh by Bakshi et al., reported Kuroki type from this region by serotype-specific PCR, but they studied only 10 randomly selected samples out of which 8 samples were positive to Kuroki type and other 2 samples were non responsive to the serotype specific primers (Bakshi et al., 2007). A study from Korea had reported the Boryong serotype being distributed throughout the country except for Cheju Island (Choi et al., 1997). In another report (Manosroi et al., 2006), Karp serotype was distributed in all regions of Thailand, whereas the Kawasaki serotype was found in the southern region of Thailand. They also reported that Karp and Gilliam serotypes were prevalent in Taiwan and Gilliam serotype was prevalent in China. Our study has helped us to identify the serotypes of O. tsutsugamushi that were prevalent in this region because
it is known that some serotypes were known to induce delayed immunological response and therefore, a delayed rise in IgM and IgG titers.

The limitation of this study was that all the N-PCR positive samples could not be serotyped by using the primers selected for the study. This signifies that other serotypes may be in circulation in this region and additional primers need to be used to detect all existing serotypes.