A COMPARATIVE STUDY ON THE DIAGNOSTIC METHODS OF SCRUB TYPHUS

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CERTIFICATE

This is to certify that the dissertation work entitled "A Comparative Study on the Diagnostic Methods of Scrub Typhus" submitted by Dr. Anisha Elizabeth Jacob, is a bonafide work done by her during the study period of her post graduation in Microbiology from June 2012 to April 2015 in our institution. This work was done under the guidance of Dr Marina Thomas, Professor, Department of Microbiology, PSG IMS & R

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1.Introduction

Scrub typhus is an infectious disease that presents as an acute undifferentiated febrile illness and could be life threatening. The agent is Orientia tsutsugamushi which is an obligate intracellular bacteria that is transmitted by larval trombiculid mite, which is the reservoir of the agent and the only life stage that feeds on a vertebrate host[8] .

Scrub typhus is endemic to the land mass bound by Japan to the north, Northern Australia to the south and the Arabian Peninsula to the west[59].Recent reports from several parts of India, including south India, indicate there has been a resurgence of the disease[79].

Scrub typhus is a disease which often goes undiagnosed due to its vague clinical symptoms and lack of a definitive protocol for its diagnosis. The clinical syndrome classically consists of fever, rash and eschar and requires laboratory confirmation of diagnosis[59]. It should be considered as a differential diagnosis in patients with acute febrile illness including those with thrombocytopenia, renal impairment, LFT abnormality, altered sensorium, pneumonitis or ARDS. A thorough search for eschar, particularly hidden areas is useful for diagnosis. Eschar may not present in a large number of cases [79].

The main stay in scrub typhus diagnosis remains serology. The oldest test is the Weil-Felix OX K agglutination reaction which is inexpensive, easy to perform and results are available overnight. [90] ELISA for the detection of IgM antibodies against Orientia tsutsugamushi offers advantages of being able to test large number of samples at a time and can be automated.[44]Indirect fluorescent antibody assay is the gold standard assay for the serological detection of antibodies in scrub typhus [47, 48].

All the currently available serological tests for scrub typhus have some limitations of which the clinician needs to be aware. Serological diagnosis based on a single acute serum sample requires a cut off antibody titer varying from 1:10 to 1:400 depending on the endemic titer. [59]

Diagnosis and surveillance of scrub typhus is challenging particularly in the absence of advanced diagnostic techniques. The availability and cost of other serological methods are a major problem in India[90]

The drug of choice for the treatment of scrub typhus is Doxycycline 200mg for 7 days. [69] In children and pregnant women Azithromycin is preferred. Rifampicin is an alternative drug but is not to be given alone to avoid the development of drug resistance. [71]

2. Aims and Objectives

2.1 AIM OF THE STUDY

To study the role of scrub typhus in undifferentiated fevers and to evaluate the serological diagnostic methods: Weil Felix and ELISA against Indirect Fluorescent Antibody assay (IFA) which is the gold standard in the diagnosis of scrub typhus.

2.2 OBJECTIVES

1. To detect the sensitivity, specificity, Negative Predictive Value and Positive Predictive Value of Weil- Felix as compared to the gold standard IFA.

2. To detect the sensitivity, specificity Negative Predictive Value and Positive Predictive Value of ELISA as compared to the gold standard IFA.

3. To observe for the seasonal variation in the scrub typhus cases.

4.To study the age and gender prevalence of scrub typhus

3. Review of literature

Scrub typhus, tsutsugamushi disease or chigger borne rickettsiosis is an acute febrile illness among humans that is caused by infection with the bacterium Orientia tsutsugamushi following the bite of infected mite vectors [2].

The term tsutsugamushi is derived from two Japanese words tsutsuga (something small and dangerous) and mushi (creature)[14]. This organism was formerly known as Rickettsia tsutsugamushi, but then it was found to be different genetically and in cell wall structure and was reclassified as Orientia[15].The word ‘typhus‘ has been derived from the Greek word ‘Typos’ for fever which means ‘fever with stupor‘ or smoke [9].

3.1 HISTORY

Scrub typhus is a historically significant disease. Evidence of this disease has been found in writings from way back in 313A.D. in China[3].The term ‘akamushi’ from which originates the Japanese term for this rickettsiosis means red chigger. The rural residents of these countries knew that the best way to avoid being infected was to avoid those areas that are infected by the arthropods [24].Medical accounts of typhus were written as early as 1536 by Cardano and in 1546 by Fracastroin. Coyttarus in 1578 was the first to suggest that typhoid and

typhus were different diseases. The illness was then later described by Hashimoto in 1810.

In 1916 Weil and Felix described the heterophile antibody agglutination of OX – 2 and OX – 19 strains of Proteus vulgaris by typhus sera. This was extended to scrub typhus by Fletcher and Lesslar in 1926. They named another agglutinated variant OX – K in honor of their friend Kingbury[9]. In 1926 the disease was distinguished from flea Borne or murine typhus and in 1936, it was described as similar to the mite borne typhus in Japan [3].

The first identification of scrub typhus was by Nagayo and coworkers in 1930. They called this organism as Rickettsia orientalis but this was then renamed as Rickettsia tsutsugamushi in 1948 and then finally Orientia tsutsugamushi in 1998. Other names for scrub typhus include chigger borne rickettsiosis, kedani (hairy mite) fever, akamushi (red mite) fever , flood fever, Japanese river fever, tropical typhus and Bush typhus[24] .

World War II has brought much new information to light regarding the subject of typhus fevers in the tropics. In the various armed forces during the Asiatic – Pacific operations, 1941 – 1945 the disease was accredited as a medical casualty producer second to

malaria and was dreaded by men [1]. During this period the US Typhus Commission brought out a programme to prevent, treat and control this disease which included development of treatments, miticides, impregnation of repellant in clothing, environmental control through burning and clearing of troop encampment areas and vaccine trails (which was ultimately unsuccessful)[2]. Even now more than 60 years after World War II there is still no effective human vaccine against scrub typhus [2].

In India, scrub typhus was recognized as typhus like fever in 1917 and there were subsequent periodic outbreaks. It is present throughout the country and reported seasonally from August to October. During the World War II it was one of the major causes of fever among the soldiers that were deployed along the Assam- India – Burma (Myanmar) border. In this area the cases were seen throughout the year but more from October to December and the mortality rate was found to be 5%.The human Gilliam prototype strain was isolated in 1943 from a soldier in this region. [2]

3.2 AGENT

They are obligate intracellular rods and are small in size, about 0.5 x 1.2 – 3.0 µm and coccobacillary in shape. They do not stain well

with Gram stain therefore other stains are preferred. The Gimenez technique is used for Rickettsia species and the Giemsa stain is used for Orientia. They retain the basic fusion when stained by method of Giemsa. They can be grown in animals, including guinea pig for Rickettsiae and mice for Orientia, as well as embryonated eggs and cell cultures.[33]

Orientia tsutsugamushi has a cytoplasmic membrane as well as the cell wall. Between the two is a clear periplasmic space. In the cytoplasm there is an electron dense ribosome rich area and a less dense area with DNA fibers [73].It has a different cell wall structure and genetic makeup from those of Rickettsia. In Orientia tsutsugamushi the cell wall lacks peptidoglycan and lipopolysaccharide and its outer leaflet is thicker than the inner leaflet as compared to Rickettsia where the inner leaflet is thicker. The bacteria is hence very soft, fragile and resistant to pencillin [15, 24]. O.tsutsugamushi lacks the gene for lipopolysaccharide synthesis but retains the pathway for polysaccharide synthesis[32].The 16s rRNA gene similarity between O.tsutsugamushi and Rickettsia is 90.2 – 90.6%[73].

The major outer membrane protein of 110, 80, 70, 60, 56, 47, 42, 35, 28 and 22 kDa are distinct from other Rickettsiacea [26]. Except

for 70- and 60-kDa proteins all others are surface proteins. The 56 kDa transmemberane protein and 60kDa heat shock protein are the most abundant [27,73]. The 56kDa protein varies among the geographical isolates of O.tsutsugamushi and it is called as the type specific antigen.[73]Amino acid homology of this protein among the strains varied from 59 – 82%. The protein has 4 variable regions which are located in the hydrophilic region of the molecule[27].

The complete genome of O.tsutsugamushi strain Boryong was recently sequenced. It comprised of circular chromosome of 2.1 Mbp and G+ C content of 30.5%.The unique feature of its genome is that upto 37.1% consists of repeat sequence. The Orientia tsutsugamushi genome additionally contains more than 400 transposases, 60 phage integrases and 70 reverse transcriptases [30, 31].

3.3 TAXONOMY

Phylum – Proteobacteria

Class - Alphaproteobacteria

Order - Rickettsiales

Family – Rickettsiaceae

Genus – Orientia

Species – Orientiatsutsugamushi[73]

The genus Rickettsia was first divided into the three groups: spotted fever, typhus and scrub typhus. Rickettsia tsutsugamushi the agent of scrub typhus was found to be distinct enough by the 16S rRNA gene sequence comparison to bring about its transfer into a new genus, Orientia and it includes only a single species, Orientia tsutsugamushi. Currently, the genus Rickettsia is made up of the typhus group (TG) and the spotted fever group (SFG) [33].

The other differences between the genus Rickettsia and Orientia are\

1. Structure of the outer envelope as revealed by electron microscope showed that the outer leaflet of the cell wall is much thicker than the inner leaflet in Orientia and the opposite occurs in Rickettsia.[34][Fig 3.1]

2. Chemically it lacks the constitutional component of peptidoglycan and polysaccharides such as muramic acid, glucosamine, hydroxy fatty acids, and 2-keto-3-deoxyoctonic acid, suggesting that neither peptidoglycan nor lipopolysaccharide is present in Orientia tsutsugamushi but these are present in genus Rickettsia. It is therefore soft and fragile and more resistant to the action of penicillin.[15]

3. The protein composition of Orientia tsutsugamushi determined by the sodium dodecyl sulfate polyacrylamide gel electrophoresis is quite different from that of the other Rickettsiae especially in the envelopes. In Orientia tsutsugamushi the most abundant protein is the 56kDa protein located on the cell surface. Other proteins are also located on the cell surface such as (80, 46, 43, 39, 35, 28 and 25kDa). Three of these are heat modifiable: 25-, 28- and 56kDa protein [26]. In Rickettsia the major antigenic proteins have size of 150 – 180, 110 – 130, 49, 32, 27.5 and 16.5 to 17.5kDa. [15].

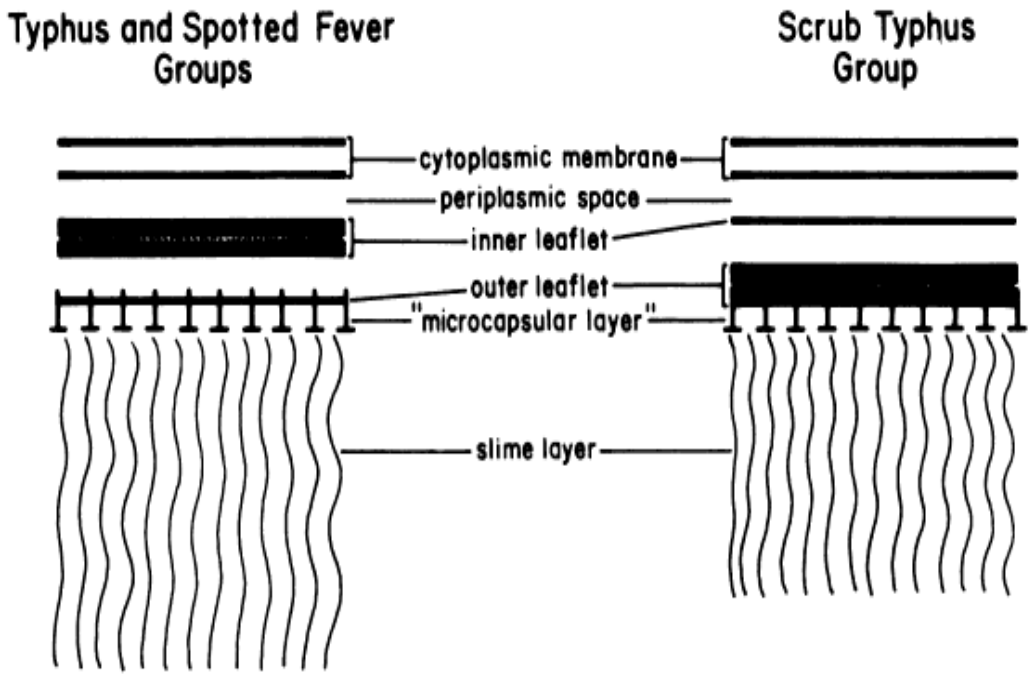


Fig 3.1: Diagrammatic representation of the cell membrane, Outer envelope and adjacent extracellular layers of rickettsiae.[34]

Various antigenic variants are recognized among the strains of O.tsutsugamushi. These antigenic strains have been distinguishable immunologically especially with strain or type specific monoclonal antibodies. The main type antigen is a 56kDa protein located on the Rickettsial cell surface. This antigen also has epitopes common among the antigenic variants.[15]

Due to the unique profile of antigenic variation, the heterogeneity among the strains is greater than that encountered in other Rickettsiales [24]. There are more than 70 different strains of O.tsutsugamushi. The three prototype strains of Orientia tsutsugamushi are Karp, Kato and Gilliam isolated from New Guinea, Japan and Burma respectively. New strains distinct from these have been found in Thailand, Taiwan, Malaysia, Japan, Korea and China [25.28]. The O.tsutsugamushi strains isolated from China, Japan, Korea are phylogenetically different from those from South East Asia which may be attributable to the vertical transmission in different mite species [29] .

The different prototypical and reference Orientia strains are given in the table below [2]

Table3.1.Prototypical and Reference Orientia strains

Remarks	Source organism ;location ; date	Strain
Original prototype strain	Human , New Guinea ; 1943	Karp
Original prototype strain	Human , Assam – Burma border; 1943	Gilliam
Original prototype strain	Human , Niigata, Japan ;1955	Kato
Strain used for vaccine trial	Human , Samar Island , Philippines; 1945	Volner (JHV)
Strain used in DFA studies by Shirai et al	Rattusrattus; KhaoYai, Khorat Plateau , Thailand; Sept 1963	TA678
Strain used in DFA studies by Shirai et al.;Karp-related strain	Rattus rajah; Chong Mek, Khorat Plateau , Thailand; Nov 1963	TA763 (Fan)
Strain used in DFA studies by Shirai et al.;Karp-related strain	Tupaiaglis; KhaoYai, Khorat Plateau , Thailand; Sept 1963	TA686
Strain used in DFA studies by	MenetesBerdmorei; Chong Mek, Khorat	TA 719

Shirai et al.;Karp-related strain	Plateau , Thailand; Aug 1963	Chon)
Strain used in DFA studies by Shirai et al	Human; Pak Tong Chai, Khorat Plateau , Thailand; Jul 1965	TH1817
-------	Human ; New Guinea ; 1943	Buie
-------	Human ; Malaya ;1934	Seerangee
-------	Human ; Dobadura , Papua New Guinea ;1943	Kostival
-------	Chigger pool ; Slavyansky district , PrimorskiKrai, Russia ; 1963	B- 15
Low murine virulence	Human ; Miyazaki, Japan ; 1971	Irie
Low murine virulence	Human; Miyazaki, Japan ; 1980	Hirano
-------	Human; Miyazaki, Japan ; 1981	Kuroki
Low murine virulence	Human ; Niigata, Japan ; 1980	Shimokoshi
-------	Human; Niigata, Japan ;1979	Ikeda
-------	Human; Niigata, Japan ;1982	Yamamoto
-------	Human; Miyazaki, Japan ; 1981	Kawasaki
-------	Rodent ; Saitama Prefecture , Japan ; 1997	Saitama

Predominant strain reported in Korea	Human; Boryong , South Chungcheong Province, South korea; 1980s	Boryong (B119)
-------	Human; Yonchon Country , Northern south Korea; 1989	Yonchon
-------	Human;Northern Territory, Australia ; Aug1996	Litchfield

3.4 EPIDEMIOLOGY

This disease is endemic to 13,000,000 km2 area of the Asia – pacific rim , extending from Afghanistan to China , Korea , the islands of the south western Pacific and northern Australia [5] .[Fig 3.2].

It is estimated that one billion people are at risk for scrub typhus and one million cases occur annually. Some Asian countries have been reporting increasing prevalence and is said to coincide with the wide spread use of beta lactam antibiotics or due to urbanization of the rural areas. [9]

The presence of spotted fevers and scrub typhus was documented from Tamil Nadu in southern India few years ago but there is little community based data available any state in India. [10]

It has been found to occur more often during the rainy seasons. In Southern India outbreaks were reported more commonly during the cooler months of the year. [6, 9].

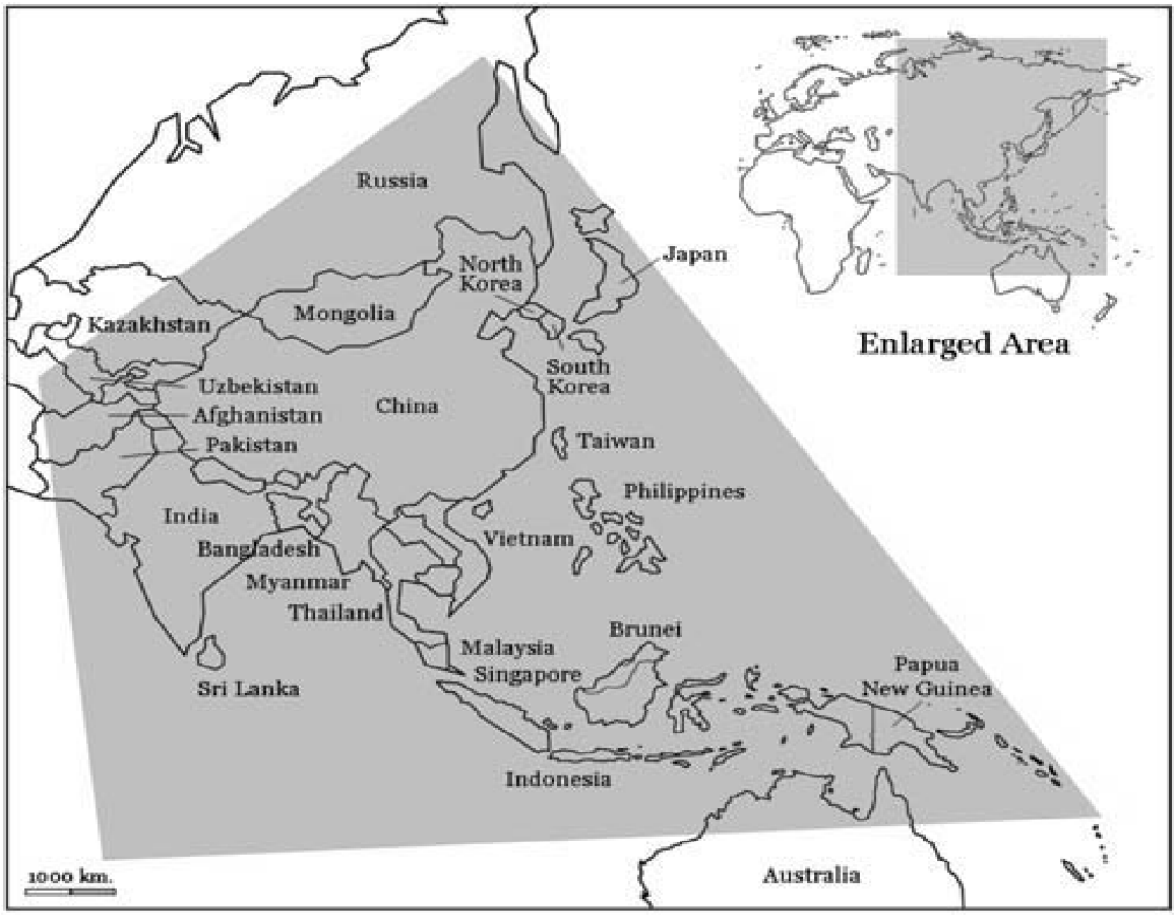


Figure 3.2.Area where scrub typhus is endemic [2]

3.5 PATHOGENESIS

The true reservoir for the infection is the trombiculid mite primarily of the genus Leptotrombidium [2,6]. The various Leptotrombidium species include *L. deliense, L. akamushi, L.scutellare, L.chiangraiensis, L.arenicola, L. imphalum, L. pallidum , L. pavlovskyi, L. fletcheri, L. gaohuensis*.[2]In India the primary vector is L.deliense[7] .

This mite is very small (0.2 – 0.4mm) and can only be seen through a microscope or magnifying glass. It is the larva or chigger stage (Fig 3.3) that feeds on the vertebrate host and is responsible for picking up and transmitting the etiological agent to the rodent host or the human incidental host. Once they are infected by feeding on the body fluids of small mammals, they maintain the infection throughout their life and adults pass the infection to the eggs in a process called transovarial transmission. The infection is then transmitted from the egg to the larvae or adult in a process called transtadial transmission [6,8] Small mammals like rats, voles and tree shrews which are mainly ground dwelling play an important role in the ecology of rickettsiosis by serving as hosts for the vector chiggers. Arboreal rodents and commensal rats as compared play a little role as they have lesser

chances in coming in contact with the ground that is infested with the vector [8]

Scrub typhus is found in a variety of habitats, mostly in those areas where the members of the Leptotrombidium deliense group of chiggers and the wild rats of the genus Rattus Rattus coexist. The habitats are characterized by changing ecological conditions brought about by man or nature and expressed by the transitional type of vegetations. The intimate association between Rats, chiggers, secondary vegetations along with O.tsutsugamushi constitute the' zoonotic tetrad'[8].

Man gets infected accidentally when he encroaches upon a zone of infected mites. These zones are made up of secondary scrub growth, which grow after clearance of the primary forest hence its named 'scrub typhus'. Mite habitats include diverse areas such as seashore, rice fields and semi deserts. [9]

The persons most commonly involved are the rural workers particularly those in agriculture and forest occupation. It can affect any age groups. Soldiers living in temporary camps are also predisposed

[6].They are not transmitted directly from person to person except by blood transfusion and organ transplantation [12].

The chiggers grasp onto a passing host and prefer to feed those body areas where the skin is thin, tender or wrinkled and the clothing is tight. They do not usually pierce the skin while feeding but insert the mouthparts down hair follicles or pores. Once attached they then inject a liquid which then dissolves the tissue around the feeding site. The liquefied tissue is then sucked up by the chigger. Large numbers of O.tsutsugamushi are found in the salivary glands of the infected chigger which are then injected into the hosts when it feeds. Once done feeding the engorged chigger drops off its host, burrows into the ground and undergoes maturation [9] . (Fig 3.4)

The organisms after entering the human body multiply locally and enter the bloodstream [12] .The bacteria can bypass the white blood cells. It can divide and multiply within the phagocytes and escape from the cell back to the circulation to continue its spread [9].

In the endothelium of small blood vessels the organisms start multiplying and releasing cytokines which damage endothelial integrity, causing fluid leakage, platelet aggregation, polymorph and monocytic proliferation and lead to focal occlusive end angitis causing

microinfarcts. This especially affects the skeletal muscles, skin, lungs, kidney, brain and cardiac muscles. This can also cause venous thrombosis and peripheral gangrene

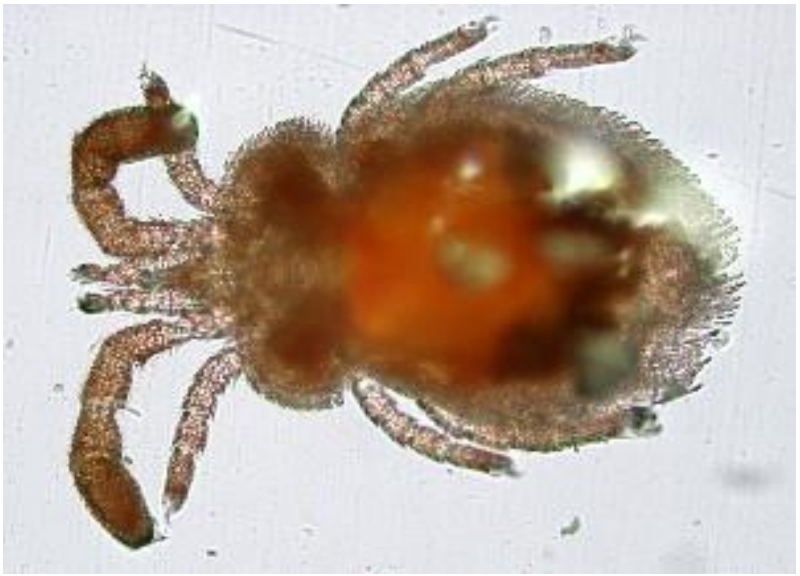


Figure 3.3 Chigger of trombiculid mite

Fig 3.4. Rattus Rattus

Fig 3.5 Scrub vegetation

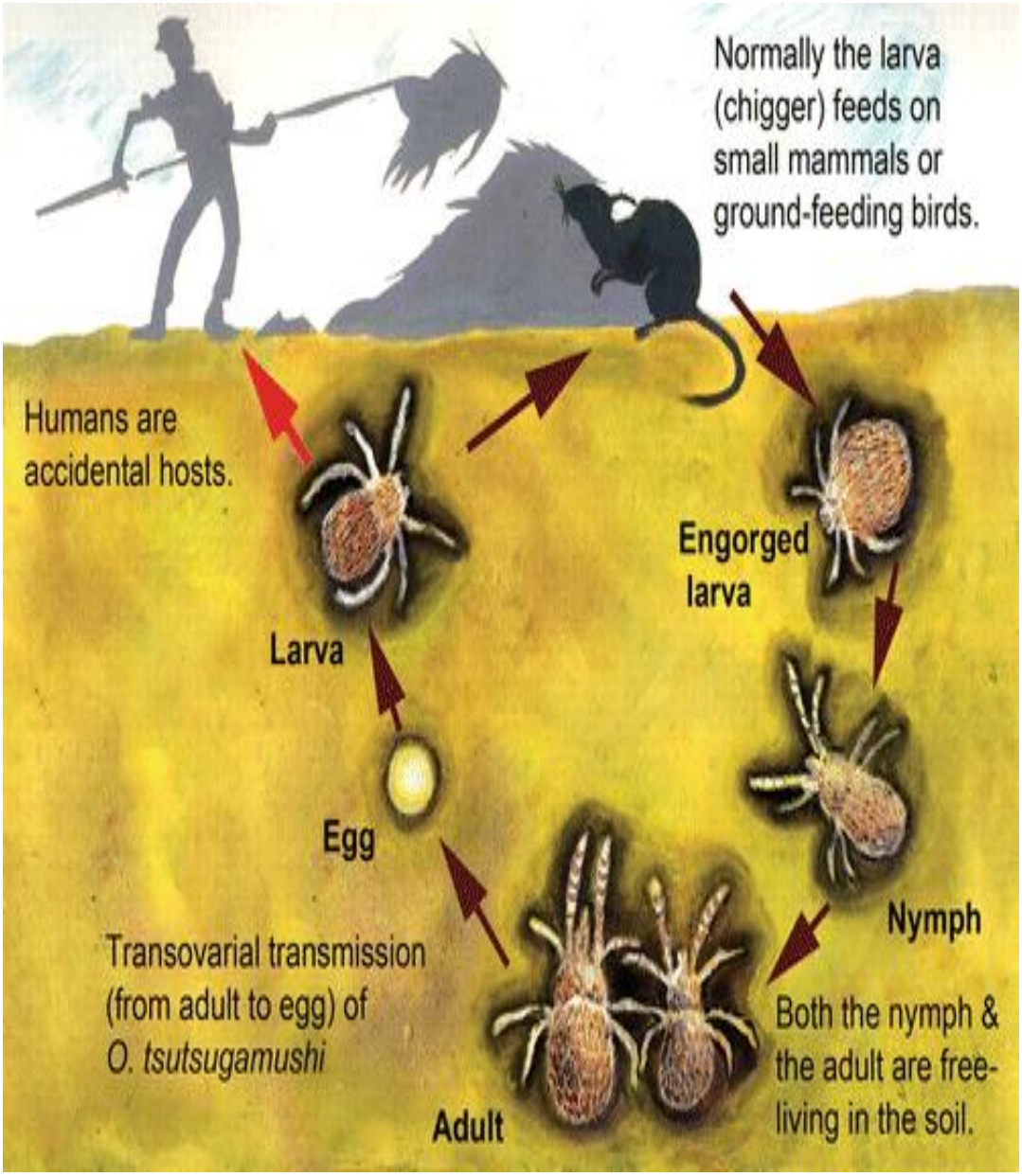


Figure 3.6 .Life cycle of Leptotrombidium mite

3.6 CLINICAL FEATURES AND PATHOLOGY

The signs and symptoms early in the course of this infection are non- specific and mimic benign viral illness, making the diagnosis difficult. Symptoms vary from mild to severe and unless there are a high index of suspicion these cases are likely to be missed. [12]

The incubation period ranges from 6 to 21 days (usually about 10 – 12 days). The onset is characterized by fever, headache, myalgia, cough and gastrointestinal symptoms. [17]

Fever of undetermined origin is the most common presentation of scrub typhus. Fever usually has an abrupt onset, high grade, occasionally with chills and morning remissions and associated with headache and myalgia. Diagnosis of scrub typhus should be considered in endemic areas with history of tick exposure.

Severe frontal headache with generalized myalgia affecting the lumbar, thigh and calf muscles are seen in some of the cases. Headache is more common in the adults as compared to children [12].

Following the onset of fever after 5 to 8 days, a maculopapular rash develops on the trunk and it may extend to the arms and legs [14].

The most useful diagnostic clue is an eschar (resembles a cigarette burn) about 5 – 20mm which is formed at the area bitten by the mite (Fig 3.5). It is seen in sites where the skin surfaces meet or where the clothing is tight such as the axilla, groin, neck, waist, and inguinal area .It is usually seen in Caucasian and East Asian patients but less seen in South Asians especially in the dark skinned [17,18]. The area bitten by the trombiculid mite initially forms a papule, which then becomes a vesicle then an ulcer and which is finally covered by a black eschar. The vicinity of the eschar is surrounded by red erythema and is typically formed at the time of manifestation of symptoms. In cases where the eschar is formed in warm, damp areas like the axilla or perineum a necrotic eschar will not be seen instead a shallow ulcer with purulent base and surrounded by a clear erythematous band may be seen. In such cases it could be easily overlooked or misdiagnosed [18].

Generalized lymphadenopathy and hepatosplenomegaly are seen in many of the cases of scrub typhus [14].

From the second week onwards some portion of the patients especially those not treated develop evidence of systemic infection [19].

Respiratory symptoms include cough and acute respiratory distress syndrome may develop [12, 14]. Pulmonary involvement is a well-documented complication of scrub typhus infection. The basic pathological process in pulmonary involvement is interstitial pneumonia with or without vasculitis[20]. At microscopy, blood vessels appear congested and surrounded by a mononuclear infiltrate[22].

Risk factors for development of ARDS include older age, thrombocytopenia, and presence of early pneumonitis. With appropriate antibiotic therapy they recover without any serious sequelae. The major cause of mortality here is a delay in the diagnosis[21].

Gastrointestinal symptoms include nausea, vomiting, abdominal pain, diarrhea with varying frequency [12]. Abdominopelvic involvement is characterized by splenomegaly but splenic infarcts are less common. Pathological findings in the liver include hepatic congestion, periportal inflammation and peripheral necrosis [23]. Gall bladder thickening may also be seen in patients with scrub typhus and could be due to acute vasculitis with perivasculitis similar to that seen in liver [14] Scrub typhus can also affect the other organs of the

gastrointestinal tract and kidneys. It can cause gastrointestinal hemorrhage and acute renal failure[14]

Neurological manifestations are quite varied. Meningoencephalitis due to scrub typhus is usually seen without focal neurological signs. Focal neurological signs but have been reported such as bilateral sixth and seventh nerve palsies, isolated sixth nerve palsy. Scrub typhus have also been associated with opsoclonus, transient parkinsonism and myoclonus [74].Pathological findings of Central nervous system involvement includes diffuse or focal mononuclear cellular infiltration of the leptomeninges, typhus nodules which are clusters of microglial cells and brain hemorrhage [23].\.

Myocardial involvement has been reported but is rare. Electrocardiographs are usually normal or they have mild nonspecific changes. Relative bradycardia has also been observed [41] .Cellular infiltration was observed in the endocardium and pericardium. Vasculitis and perivasculitis in the myocardium induce cellular infiltration along with hemorrhage and edema of the interstitial tissue. Cardiomegaly may be seen and it is usually reversible.[14]

In some cases the patient develops Multi Organ Dysfunction Syndrome (MODS).Due to the varied clinical presentation of scrub typhus the diagnosis is often missed or delayed[19].

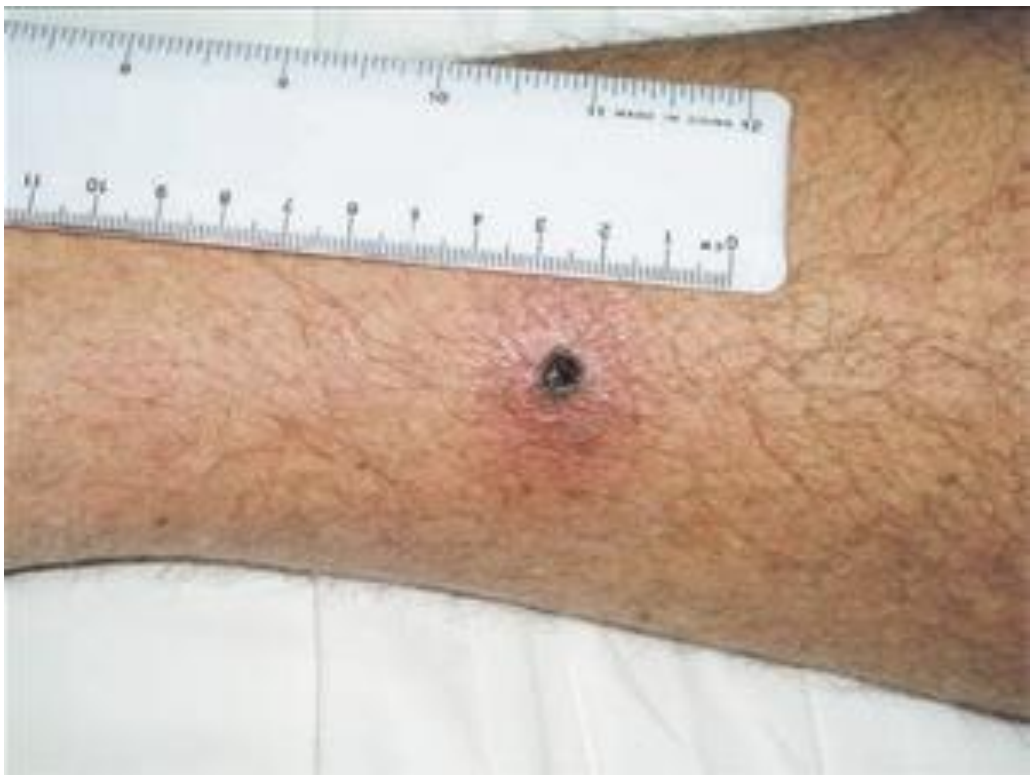


Fig 3.7 .Eschar[91]

3.7 DIAGNOSIS OF SCRUB TYPHUS

The clinical symptoms and signs of scrub typhus may be difficult to differentiate from other endemic diseases such as typhoid fever, leptospirosis, dengue fever and murine typhus [61]. The diagnosis of scrub typhus therefore depends on clinical suspicion thereby prompting the clinical to request for the appropriate laboratory investigations [59]. Although the disease may be self-limiting, an accurate and prompt diagnosis is necessary to reduce the duration of fever and prevent complications. The diagnosis of scrub typhus mainly relies on serology of which IFA is the gold standard [61]

3.7.1 ISOLATION OF ORIENTIA

Orientia tsutsugamushi can be isolated from the buffy coat of heparinized blood, defibrinated whole blood, triturated clot, plasma, necropsy tissue, skin biopsy and arthropod samples.[47]

Whole blood from the patients can be inoculated intraperitoneally into white mice, which are then observed for signs of illness or death. The organism can also be observed by Giemsa stained impression smears taken from the surface of the spleen or peritoneum[41].Inoculation into guinea pig was done but now mouse is the species of choice for O.tsutsugamushi. Meadow voles are also

susceptible to the infection but because of their reduced availability they are not commonly used. [47] Though animal inoculation is more laborious and expensive [15] they are preferred in situations which require the isolation of the organism from post mortem tissues which are contaminated by other bacteria and also to remove the contaminating Mycoplasmas from the cell culture for rickettsia. [55]

Embryonated eggs were widely used in the past but now cell culture techniques have replaced them. Cell culture is the most widely used method in order to isolate Orientia from the clinical samples.

Isolation of Orientia requires Biosafety level – 3 facilities for culture on cell monolayer and the median time for positivity is 27 days [37]. . O.tsutsugamushi can be cultivated in the yolk sac of developing chick embryos and can be grown in the culture of cell lines such as HeLa, BHK, Vero and L929 cell lines [15].

Early on Primary monocyte culture was used for the isolation of R.rickettsii from blood[56.] Later an L929 mouse fibroblast cell monolayer in tissue culture was introduced which was used for the isolation of R.rickettsii and O.tsutsugamushi from blood.[63] Most of them grow in the perinuclear cytoplasm of host cells and then release themselves by pushing out the host cytoplasmic membrane from the

inside like budding seen in enveloped viruses. The budding Orientia accumulate at high density at the host cell surface. The budding time is about 9 to 18 hrs in well adapted cells[15].

The isolation rates vary but are usually less than 50% even when the test in done under the very good conditions by an experienced worker[41].

Table 3.2 .Comparison of isolation methods of scrub typhus [59]

Assay	Comments	Setting	Ease	Time	Cost / sample	Specificity	Acute sensitivity	Format
In vitro isolation (cell culture)	• Isolation of BSL3 agent • Requires infrastructure • Bio containment issues • Retrospective diagnosis	BSL3 reference lab	+	7 – 60 days	+++++	+++++	+	Isolation
Mouse inoculation	• Technically demanding • Isolation of BSL3 agent • Requires animal facilities • Bio containment issues • Retrospective diagnosis	BSL3 reference lab	+	5 – 30 days	+++++	+++++	+	Isolation

3.7.2 SEROLOGICAL METHODS

All the currently available serological tests for scrub typhus have their limitations of which the clinician needs to be aware though they are used widely. Greater than or equal to the 4 fold rise in titer between two consecutive samples is considered positive, but such a diagnosis is retrospective and cannot guide in the initial treatment. If the diagnosis is to be based on a single acute serum we would be requiring a cut off antibody titer. The cut offs from 1:10 to 1:400 is quoted often with little evidence and without establishing the titers in a healthy population [42].

3.7.2a Weilfelix agglutination test

In 1916 Weil and Felix described the heterophile antibody agglutination of OX – 2 and OX – 19 strains of Proteus vulgaris by typhus sera. This was extended to scrub typhus by Fletcher and Lesslar in 1926. They named the other agglutinated strain of Proteus mirabilis Ox – K in honor of their friend Kingbury.[9,47]

The Weil Felix test was the first to be used and involves non specific antigen. The antigen used is the non-specific variant of non-motile strains of Proteus vulgaris designated as OX – 19, OX – 2 and Proteus mirabilis OX – K. They are agglutinated by the antibody that is

developed in the blood of patients suffering from Rickettsial diseases[39].

Table 3.3 Weil Felix test agglutination reactions

OX – K	OX – 2	OX - 19	Rickettsial infections
--	+/++++	++++ /+	Rocky mountain spotted fever
--	+	++++	Epidemic typhus
--	+	++++	Murine typhus
--	--	--	Brill – Zinsser disease*
++++	--	--	Scrub typhus
--	--	--	Rickettsialpox
--	--	--	Q fever

* = A positive OX – 19 reaction is occasionally seen

++++= A fourfold / greater rise in titer -- = no reaction

The Weil- Felix test is of no diagnostic value in separating Murine typhus, epidemic typhus and Rocky Mountain Spotted Fever; it only indicates the presence of Rickettsial infection. Presumptive evidence is obtained earlier as Proteus agglutinins appear 4 – 5 days after the onset and rapidly rise by the 10^{th} – 12^{th} day. Antibodies decline to a non-diagnostic level 1- 4 months later[39].The antibodies being detected are mainly of IgM type[47].

Preparation of antigen includes the following method

1. First streak the Proteus vulgaris/mirabilis stain on dry agar that contains 0.5% phenol to develop and maintain non motile variants.

2. Then suspend the growth from the 18 – 24 hr culture into 0.85% saline and adjust the turbidity to the tube 3 of the McFarland nephelometer scale. The emulsion should not be heated as heating it at 56^0C can destroy its agglutinability.

3. It can be used as live antigen or killed by adding 0.5% formalin.

4. It is then stores under refrigeration. On prolonged storage it becomes hyperagglutinable. [39]

Procedure

1. Prepare serial 2 fold serum dilution ranging from 1:20 – 1:640.
2. Mix 0.5ml of each dilution with 0.5 ml of Proteus OX – 19 antigen
3. Repeat set up with OX – k and OX – 2 antigens.
4. To permit the correct reading, prepare turbidity controls as shown below :

0.125ml	0.25ml	0.5ml	Antigen
0.975ml	0.75ml	0.5ml	Saline
3+	2+	0	Agglutination

5. Incubate test and control tubes at 50^{o}C in water bath for 18 – 24hrs with tubes submerged to the depth of $1/3^{rd}$ of the column of liquid so as to create circulation of serum antigen mixture by temperature differential.

6. After inoculation allow the tubes to cool to room temperature and read the degree of agglutination as 0, 1+, 2+, 3+ and 4+ by comparing supernatant fluid with that of the control tubes.

Interpretation

The titer is determined as the reciprocal of the highest dilution at which the 2+ or greater reaction is observed.

A fourfold or greater rise in antibody titer between the acute and convalescent sera specimen is essential for presumptive diagnosis. The titer of a single specimen is taken positive only if it is more than 1:160.A low static tire could suggest a past proteus infection [39].

A positive Weil Felix reaction was seen only after the fifth day of the illness and not before the appearance of the characteristic rash, so this test is of little value in the early diagnosis. [40]

Weil Felix is not specific nor a very sensitive test. A positive Weil Felix test is also seen with louse borne relapsing fever, leptospirosis or other febrile illness and also with Proteus urinary tract infections. Only less than 50 % of those with well documented scrub typhus infection have a rising or strongly positive Weil Felix titer. A reinfection with scrub typhus infection does not cause a second rise in OX K agglutinins[42]

In a study conducted in South India, they tested 125 serum samples with both Weil Felix and ELISA tests. Here the sensitivity for Weil Felix OX-K was 30% at a titer breakpoint of 1:80, but the specificity and positive predictive value were 100%. At a breakpoint of 1:20, the sensitivity was 61%, the specificity was 94%, and positive predictive value was 84%. At a breakpoint of 1:40, the sensitivity was 49%, the specificity was 96%, and positive predictive value was 88%.[43]

Another study from the same institution showed that Weil Felix was found to have a sensitivity of 43% and specificity of 98% for titers 1:80 or more [75]. However, another study from Sri Lanka stated that Weil Felix has a low sensitivity (33%) in diagnosing acute Rickettsial infections and low specificity with a positive titer of 1:320 seen in 54% of healthy volunteers and 62% of non rickettsial fever patients[76].

A study done in Thailand showed that test was insensitive with the overall sensitivity being about 33% but reasonably specific at about 93.3% and 97.3% respectively at the cut off titers of 1:160 or 1:320.The reduced sensitiviy could be due to relatively late appearance of appropriate antibodies (agglutinating antibodies are detectable only in the second week of illness), prior antibiotic treatment leading to

suppression of antibody production or their absence in case of reinfections with scrub typhus which is common in the hyper endemic areas in Asia[61].

3.7.2b ELISA

It was first introduced for the use in detection of antibodies against Rickettsia typhi and Rickettsia prowazekii and then later adapted for use in Rocky Mountain Spotted Fever and Scrub typhus[47].

In the original approach "paper ELISA" was proposed to detect anti – O.tsutsugamushi antibodies. Here the initial steps are similar to IFA and then an anti-human IgG peroxidase conjugate and substrate saturated filter paper are used on which the reaction can be visualized [51]

For the antigen preparation in ELISA due to safety issues associated with the culturing of live rickettsia, the recombinant 56-kDa immunodominant protein from O.tsutsugamsuhi is used to develop serological ELISA for scrub typhus. Large quantities of this can be prepared using E.coli without needing BSL 3 facilities [52].

A variety of Enzyme linked immunoassays have been developed for the diagnosis of Rickettsial infections. They offer certain advantages over the other serological assays as they can be automated and therefore not dependent on the user interpretation of result. Also a large number of results can be run at a time [44].This procedure is also highly sensitive and reproducible and one can differentiate between IgG and IgM[47]. Disadvantages are its decreased specificity over other assays and they are not practical for small number of sera[44].As shown by one study the sensitivity in using Native Karp ELISA was 88%, Native Gilliam ELISA was 88% and r56 Elisa was 86%. The Specificity was 90, 94 and 84% in that order for the same [52]. ELISA when performed with the 56 kDa recombinant antigen showed a sensitivity and specificity of about 90 %. [43]

3.7.2c Indirect Fluorescent Antibody (IFA) Assay

Indirect fluorescent antibody assay is the gold standard assay for the serological detection of antibodies in scrub typhus[47, 48]. This was first described in 1963 by Bozeman et al[45] and has then undergone modifications to allow the use of smaller amount of serum and antigens and is now currently known as micro assay[49].

The micro IFA provides us with the advantage that it can simultaneously detect antibodies to a number of Rickettsial antigens with the same drop of serum sample to a single well which contains the multiple antigen dots (upto 9 different antigens can be tested). IFA allows the detection of IgG and IgM, or both.[47]. IFA most frequently uses antigen from three serotypes: Karp, Kato and Gilliam. But there has been a lot of antigenic variation in different areas [2]. In a single field from Malaysia 8 different serotypes were found in mites [53]. Therefore in Japan the Infectious Disease Surveillance Center has recommended a way approach for the diagnosis of scrub typhus. Firstly that local strains are to be included in the IFA depending on the area and secondly that PCR of the blood clot is to be done on all specimens [54] (buffy coat is preferred).These recommendations are not applied much outside that of Japan[45].

The identification by IFA of specific IgM antibodies to the various species of rickettsia gives a strong evidence of recent Rickettsial infection but this may be obscured by the prozone phenomenon[50].

Rheumatoid factor could affect this technique therefore rheumatoid factor absorbent is to be absorbed before IgM

determination. Sera are diluted with PBS with 3% non-fat powdered milk to avoid nonspecific fixation of antibodies. IFA uses epifluorescence to visualize fluorescein linked anti – human antibody conjugate to detect the presence of scrub typhus specific antibody bound to the smears of scrub typhus antigen[47]. The persistence of detectable antibodies in patients with scrub typhus is controversial as old reports have shown that they persist for many years but recent reports over a period of two years show there is an annual revision rate from more than 1:50 to titers less than 1:50 in 61% of the patients [47].This could be because of the variable rates of reinfection and strain heterogenicity.

In case of primary infection by O.tsutsugamushi, a significant antibody titer is observed at the end of the first week, concomitant with the detection of IgM antibodies but the IgG antibodies appear at the end of the second week. If there is a reinfection, the IgG antibodies are detectable by day 6, with the IgM antibody titers being variable [47].The sensitivity and specificity of IFA at various titer are; at titer of $\geq$1:100 84% and 78%, $\geq$1:200 70% and 92%, at $\geq$1:400 48% and 96%[77].

3.7.2d Indirect Immunoperoxidase

This test was developed as an alternative test to Indirect Fluorescent Antibody assay. It was first used for the diagnosis of scrub typhus and was then extended to Rickettsia conorii and Rickettsia typhi[47]. In this method one does not require a fluorescent microscope as the fluorescein dye is replaced by peroxidase [57]. Another added advantage of this method is that it provides us with a permanent slide record[47]. It is shown that the sensitivity and specificity by the immunoperoxidase assay is same as that of IFA [58].

3.7.2e Rapid point of care tests

Different rapid bed side tests have been developed based on serology such as the Integrated diagnostic Dip – S – Ticks [59].This is a dot - blot enzyme linked immunosorbent assay (dot – ELISA)for the detection of O.tsutsugamushi specific antibody [60]. In this method minute amount of antigen is adsorbed on to the nitrocellulose filter paper.

The patient's serum and enzyme conjugated antibody is added and incubated. A precipitable, chromogenic substrate is then added which causes the formation of a coloured dot on the solid phase

which is then read visually. This is a simple test and does not require a microscope and therefore practical for use in village settings .This assay has shown to have a good sensitivity (3 dots 81.2%, 2 dots 86.3%, and 1 dot of 94.0%)and specificity (3 dots 100%,2 dots 98.7% and 1 dot 98.7%) [61].

Besides this we have other rapid detection kits like the Scrub typhus cassette format rapid flow assay(RCT), and scrub typhus IgM and IgG Rapid Immunochromatographic Assay (PaBio, Brisbane, Australia). Though these have reached the market yet they are far from reach in most developing countries because of the high cost[62].

3.7.2f Western immunoblot assay

Western immunoblot assay with sodium dodecyl sulfate-gel-electrophoresed and the electroblotted antigen is a useful tool for seroepidemology and confirmation of the serologic diagnosis obtained by conventional tests. It is very handy when it comes to differentiating true positive from false positives created by the cross reacting antibodies[47].The western immunoblot assay is the most specific tool in determining the true prevalence of Rickettsial diseases[47].

3.7.3MOLECULAR METHODS

The current methods of isolation are not appropriate for routine diagnosis and the current gold standard IFA is imperfect. There is therefore an urgent need for newer isolation methods[59].The molecular methods include as below

3.7.3a Polymerase chain reaction

In a study in Korea, PCR of eschars on those patients who were IFA negative but with typical eschars showed that six out of the seven patients had O.tsutsugamushi DNA positive. The primers used were based on the nucleotide sequence encoding the 56-kDa major wall protein antigen of the O. tsutsugamushi Gilliam species.

The primers 34 (5′-TCAAGCTTATTGCTAGTGCAATGTCTGC-3′) and 55 (5′-AGGGATCCCTGCTGCTGTGCTTGCTGCG-3′) were used in the first PCR, and the nested PCR primers 10 (5′-GATCAAGCTTCCTCAGCCTACTATAATGCC-3′) and 11 (5′-CTAGGGATCCCGACAGATGCACTATTAGGC-3′) were used in the second PCR[64].Another study showed that out of 20 patients with fever three had positive O.tsutsugamushi PCR but negative

serology[65]. The high cost of resources and training required for PCR makes it impractical in many areas. Another problem faced with PCR for scrub typhus is the sample to be selected. The use of whole blood, buffy coat and eschar has been attempted[59].

3.7.3b Loop mediated isothermal amplification

In this technique amplifying DNA is done using three specially designed primer pairs and the Bst DNA polymerase. Here there is no DNA extraction procedure and the entire amplification reaction is done in one temperature so one requires only a Water bath or heating block. The final reaction is then read visually and no special equipment is required[59].One study has shown that LAMP could detect levels as low as 14copies/µl as compared to 3 copies/µl for PCR [66]

3.8 TREATMENT

In tissue cell cultures , O.tsutsugamushi are found to be susceptible to tetracycline, doxycycline, minocycline, chloramphenicol and rifampicin[67].Quinolones like norfloxacin , ciprofloxacin and ofloxacin were found to be only moderately active and beta lactams and nalidixic acid were found to be inactive[68].

Scrub typhus responds more quickly to antibiotics than other Rickettsial diseases and the patients become afebrile in about 24 to 36 hrs after starting the antibiotic therapy[69]. If the fever does not subside in about 48hr then the diagnosis of Scrub typhus is highly unlikely [70].

The first effective antibiotic is Chloramphenicol (2g/day) in the treatment of scrub typhus. Currently the drug of choice is Doxycycline 200mg daily for 7 days. Shorter treatment may be curative but is associated with relapse [69].However one study found short course therapy to be effective .In this multicenter trial, 116 patients with scrub typhus were randomized to receive seven days of tetracycline (500 mg four times daily) or three days of doxycycline (100 mg twice daily). The patients were followed for four weeks after the completion of therapy. The cure rate was 100 % in the tetracycline group, and 94 % in the doxycycline group. There were no relapses with either regimen [72].

For small children and pregnant women azithromycin is the drug of choice. Rifampicin is also used as an alternative drug but it should not be used alone because of chances of development of resistance. It can be used in combination with azithromycin or

doxycycline in areas where there is poor response to doxycycline alone [71].

3.9 PREVENTION

- Mite control – this includes avoidance of mite infected areas , wearing protective clothing , personal prophylaxis by impregnating clothes with miticidal chemicals (permethrin and benzyl benzoate) by applying mite repellents on exposed skin surfaces (such as Diethyl toluamide) and by applying chlorinated hydrocarbons to the ground and vegetations in focal areas (such as lindane, Dieldrin and chlordane).
- Chemoprophylaxis – weekly once dose of doxycycline 200mg is found to be effective.[71]
- Vaccines – As of now there is no effective vaccine for scrub typhus. There is large amount of antigenic variation in Orientia tsutsugamushi and one strain does not confer immunity to another. The scrub typhus vaccine should be capable of providing protection to the local strains [71]

Fig 3.8 Personnel protective clothing

4. Materials and Methods

4.1 **STUDY PERIOD**:

The study was done for a period of about 2 years from October 2012 to august 2014.

4.2 **STUDY TYPE**:

Screening test evaluation of Weil Felix and ELISA as compared to the gold standard Indirect Fluorescent Antibody assay (IFA) and a descriptive study of scrub typhus

4.3 **SAMPLE SIZE**:

We tested 633 suspected cases of scrub typhus patient samples by Weil Felix / ELISA. For comparison of Weil Felix and ELISA against the gold standard IFA, 50 IFA scrub typhus positive and 50 IFA negative samples from suspected scrub typhus cases were taken.

4.4 **STUDY POPULATION**:

Among the patients who were visiting or admitted in PSG hospital with fever, those who were clinically suspected of scrub typhus were included in this study.

4.4.1 Inclusion criteria: consecutive blood samples from suspected cases of scrub typhus were included in the study.

4.4.2 Exclusion criteria: Samples that were lysed, icteric or turbid were not included.

4.4.3 Ethics approval: Approval was obtained from the institutional Ethics committee at the start of the study and was renewed periodically during the study period

4.4.4Data collection method: Details of the patients were entered into the data collection form. The details included the name, age, gender, address and occupation.

4.5 SAMPLE **COLLECTION**:

From the suspected cases of scrub typhus patients, three ml of blood was collected in red (for OP patients) or yellow capped (for ward) BD vacutainer tubes.

4.6 **CONTROLS**:

50 controls were included in this study. Control group comprised of patients with fever but not suffering from scrub typhus.

These samples were processed in the same steps as the samples in the study group.

4.7 **SAMPLE PROCESSING**

The BD vacutainer tubes with the patients sample were centrifuged at 6000rpm for 15min. One ml of the clear serum on top was transferred to small vials. These were then labeled and numbered and the details were entered into the data collection form.

As and when the samples were obtained Weil Felix test was done and ELISA was done weekly. It was then stored at -80^{o}C. When sufficient sample size had been obtained these samples were thawed and IFA was done on the samples.

4.7.1 Weil Felix

Weil Felix test was done using the PROGEN antigen suspension manufactured by the Tulip Diagnostics (p) LTD, Goa.

Principle:

The smooth, killed, stained PROGEN antigen suspensions are mixed with the patient's serum. Antibodies produced due to Rickettsial infection if present in the patient serum will react with the stained PROGEN antigen suspension to produce an agglutination reaction. No agglutination indicates the absence of cross reacting antibodies.

Materials Required**:**

-Test tubes (12 mm x 75 mm), 8 for each test

-Test tube rack

-Pipettes/ Micropipettes,

-Physiological saline

-Incubator (37°C),

-Progen OXK Antigen Suspension

-Serum from patient suspecting scrub typhus.

Procedure**:**

- In a test tube rack 8 test tubes were taken and arranged in a row, and labeled 1 to 8. The Sample number was written on the first tube. Into the first tube 1.9 ml of physiological saline was added.
- To each of the remaining tubes (2 to 8) 1 ml of physiological saline was added.

- To tube No. 1, 0.1 ml of serum sample to be tested was added and mixed well.
- From tube No. 1 to tube No.2, 1.0 ml of the diluted serum sample is transferred and mixed well.
- Then 1.0 ml of the diluted serum sample from tube No. 2 is transferred to tube No.3 and mixed well. This serial dilution is then continued till tube No. 7.
- From Tube No. 7, 1ml of the diluted serum sample was discarded.
- Now the dilutions of the serum sample achieved from tube No. 1 to 7 respectively in each set is were follows: 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, and 1:1280. Tube No. 8 was kept as the negative control.
- To all the tubes (1 to 8) one drop of well mixed OXK PROGEN antigen suspensions from the reagent vials were added and mixed well.
- The tubes were then incubated at 37^{o}C for approximately 18 hours.
- After the appropriate incubation period the tubes were observed for clearing and granular clumping.

Interpretation of results:

In positive reaction there was clearing and granular agglutination, in negative reaction with physiological saline as negative control a button was formed with no clearing. Titres of ≥1:160 was taken as significant. However lower titres may be seen in the acute phase of the infection and this was noted.

Positive reaction can occur due to previous vaccinations, anamnestic response, antibiotic therapy, narcotic addiction and other diseases such as malaria, infectious mononucleosis, typhoid, brucellosis, tuberculosis, liver disease, auto agglutinations as well as urinary infections by Proteus. Therefore results must be judged on the context of clinical findings.

4.7.2 Enzyme linked Immunosorbent Assay (ELISA)

The ELISA test was done using the Scrub typhus Detect™ IGM ELISA system manufactured by InBios International, Inc., USA.

Principle**:**

The Scrub Typhus Detect ELISA system for IgM Test is a qualitative ELISA for the detection of IgM antibodies to O. tsutsugamushi in serum. Wells of each plate have been coated with O.

tsutsugamushi derived recombinant antigen mix. During testing, the serum samples are diluted in InBios sample diluent and applied to each well. After incubation and washing, the wells are treated with polyclonal Goat antihuman IgM antibodies labelled with the enzyme horseradish peroxidase (HRP). After a second incubation and washing step, the wells are incubated with the tetramethylbenzidine (TMB) substrate. An acidic stopping solution is then added and the degree of enzymatic turnover of the substrate is determined by absorbance measurement at 450nm. The absorbance measured is directly proportional to the concentration of IgM antibodies to OT present. A set of positive and negative controls are provided as internal controls. These are provided to monitor the integrity of the kit components.

Materials Required**:**

The Scrub Typhus Detect ELISA system for IgM (1 x 96 Wells)

contains sufficient reagents for 96 wells.

Each kit contains the following reagents:

-Scrub Typhus ELISA Plate with wells coated with O. tsutsugamushi derived recombinant antigen in each well

-Sample Dilution Buffer for Scrub Typhus

-Scrub Typhus IgM Positive Control

-Scrub Typhus Negative Control

-Ready to Use Enzyme Conjugate-HRP for Scrub Typhus IgM

-10X Wash Buffer

-EnWash

-Liquid TMB Substrate

-Stop Solution.

Other materials required but not provided are

-Microtiter plate reader capable of absorbance measurement at 450 nm

-Biological or High-Grade Water

-37^0C Incubator

-Pipettes,

-Timer and Human test serum.

Procedure**:**

- The samples were allowed to reach room temperature (~25°C) and mixed thoroughly by gentle inversion before use.
- Positive, negative controls and unknowns were assayed in duplicate.
- The number of sera to be tested was determined and organized according to the "Example for Sera Application" provided in the next page

- Test sera were diluted to 1/100 by using the provided Sample dilution Buffer for Scrub Typhus (such as 4µl of serum plus 396µl of Sample dilution Buffer for Scrub Typhus) and mixed well.
- To the marked Scrub Typhus ELISA plate 100µl per well of the 1/100 diluted test sera and controls were added
- The plate was covered with parafilm and incubated at 37°C for 30 minutes in an incubator.

Example for Serum Sample Application												
	1	2	3	4	5	6	7	8	9	10	11	12
A	PC	S# 7	S# 15	S# 23	S# 31	S# 39	S# 47	S# 55	S# 63	S# 71	S# 79	S# 87
B	NC	S# 8	S# 16	S# 24	S# 32	S# 40	S# 48	S# 56	S# 64	S# 72	S# 80	S# 88
C	S# 1	S# 9	S# 17	S# 25	S# 33	S# 41	S# 49	S# 57	S# 65	S# 73	S# 81	S# 89
D	S# 2	S# 10	S# 18	S# 26	S# 34	S# 42	S# 50	S# 58	S# 66	S# 74	S# 82	S# 90
E	S# 3	S# 11	S# 19	S# 27	S# 35	S# 43	S# 51	S# 59	S# 67	S# 75	S# 83	S# 91
F	S# 4	S# 12	S# 20	S# 28	S# 36	S# 44	S# 52	S# 60	S# 68	S# 76	S# 84	S# 92
G	S# 5	S# 13	S# 21	S# 29	S# 37	S# 45	S# 53	S# 61	S# 69	S# 77	S# 85	S# 93
H	S# 6	S# 14	S# 22	S# 30	S# 38	S# 46	S# 54	S# 62	S# 70	S# 78	S# 86	S# 94

- After the incubation was complete the strips were washed six times with the 1X Wash Buffer using an automatic plate washer. In each wash cycle for all plate washing, 300µl per well of 1X Wash Buffer was used.
- Per well, 100µl of Ready to Use Enzyme-HRP Conjugate for Scrub Typhus IgM was added into all wells by using a pipette.
- The plate was then covered with parafilm and incubated at 37°C for 30 minutes in an incubator.
- After the incubation, the plate was again washed 6 times with automatic plate washer using 1X wash buffer, 300 µl per well.
- Into all wells, 150µl Of EnWash was added by using a pipette.
- The plate was incubated at room temperature (20-25°C) for 5 minutes without any cover on the plate.
- After the incubation 100 µl per well of Liquid TMB substrate was added into all wells by using a pipette.
- The plate was then incubated at room temperature (20-25°C) in a dark place (or container) for 10 minutes without any cover on the plate.
- Lastly 50 µl per well of Stop Solution was added into all wells by a pipette and incubated at room temperature (20-25°C) for 1 minute without any cover on the plate.

- After the incubation, the Optical Density (OD) 450nm value was read with a Microtiter plate reader.

Results**:**

Calculation of the cut off value**:**

Calculation of the cut- off value was determined by the average OD plus three times of the Standard Deviation(SD) of normal human serum and human sera with unrelated infections.

Interpretation of the results**:**

1. Samples with the spectrometric reading > cut – off were considered to be “Reactive” and those below this criterion were considered to be “Non-Reactive”.

Ensuring Assay performance**:**

The results on the table below were obtained using provided positive and negative control to calculate discrimination capacity of the assay: Non-fulfillment of these criteria is an indication of deterioration of reagents or an error in the test procedure and the assay was then repeated.

Factor	Tolerance
Negative Control(NC) OD	< 0.200
Positive Control(PC) OD	> 0.500
Discrimination Capacity ($R_{PC}/_{NC}$)	≥ 5.0

4.7.3 Indirect fluorescence Antibody assay (IFA)

The IFA test was done using Orientia tsutsugamushi IFA IgM antibody kit manufactured by the Fuller Labs , California , USA.

Principle

The IFA slides in this kit utilize 4 strains (Gilliam, Karp, Kato and Boryong), purified from in vitro propagation and presented as four antigen dots within each slide well. Patient sera are diluted at least 1:64 in an adsorbent suspension and incubated in the individual slide wells to allow reaction of serum antibody with the Orientia. The slides are then washed to remove non-reactive serum proteins, and a fluorescence

conjugate is added to label the antigen-antibody complexes. After further incubation, the slides are washed again to remove non-reactive conjugate. The resulting reactions can be visualized using standard fluorescence microscopy, where a positive reaction is seen as small sharply defined fluorescent rod forms dispersed within a red-counterstained background matrix. A negative reaction is seen as either counterstained (red) background or fluorescence different from that seen in the Positive Control wells.

Materials required

The kit contains

-Substrate Slides - 10 x 12-well masked slides containing 4 acetone-fixed antigen

strains (Gilliam, Karp, Kato and Boryong) in each well.

-IgM Conjugate, 2.5 mL

-IgM Serum Diluent, 10 mL

-Positive Control, 0.5 mL

-Negative Control, 0.5 mL

-Mounting Medium, 1 mL

-PBS, 1 liter

Materials Required But Not Supplied

- Purified (distilled or deionized) water

-Clean 250 or 500 mL wash bottle for PBS

-Wash bath with slide rack

-Test tubes or microtiter equivalent for manual dilutions

-Precision pipette(s) for making dilutions and delivering exactly 20 µL per slide well

-24 x 50 mm glass cover slips

-Fluorescence microscope with filter system for FITC(maximum excitation wavelength 490 nm, mean emission wavelength 530 nm) and 400X magnification

-37ºC water bath or incubator

-Humidity chamber for slide incubation steps

Procedure

Preparation of samples and reagents

1. Wash Buffer was prepared by adding contents of PBS packet to 1 liter purified water and mixing thoroughly.

2. The patient's sample was diluted by adding 10µl of the patient sample to 150µl of the MIF IgM sample diluent giving a dilution of 1:16. This was then diluted further by taking 50µl from the above and

adding to 150µl of the Wash buffer giving the dilution of 1:4. The final dilution of the patient sample was 1:64.It was mixed well and this mixture was allowed to incubate at least 20 minutes at ambient temperature. These treated samples were centrifuged and the supernatant serum was used for testing.

3**.** This 1:64 dilution was referred to as the screening dilution.

Assay procedure

1. Serial 2-fold dilutions in Wash Buffer of the Positive Controls was prepared to include 1 dilution above and 1dilution below the stated endpoint (1:512). All controls are pre-diluted and bottled at 1:64.

2. For each serum sample, 20 µL was added to one slide well and the location is recorded for later reference. For each assay the Positive Control and the serial dilutions of the Positive Control prepared in step 1 were included. Also one drop of Negative Control was added to one well. Samples were applied to the top or bottom edge of the well, avoiding the mid-section containing the antigen microdots.

3. The slides were placed into a humidity chamber and incubated for 30 minutes at 37º± 0.5ºC.

4. The slides were removed from the humidity chamber and rinsed with a gentle stream of PBS from wash bottle. One row of wells was washed at a time to avoid mixing of specimens.

5. To each slide well 1 drop (20 µL) IgM Conjugate was added, then the slides were placed in the humidity chamber for 30 minutes incubation at 37º± 0.5ºC. Incubation was in the dark to protect the photosensitive conjugate.

6. The slides were washed as in steps 5-6.

7. To each slide 2 – 3 drops of the mounting medium is added and the cover glass is applied.

8. The stained substrate slides were read at 400X magnification under the fluorescent microscope in a dark room, comparing each well to the visual intensity and appearance of the Positive and Negative Control wells. Slides were then stored at 2 - 8ºC in the dark.

Interpretation of results

A positive reaction appeared as bright staining (at least 1+) of short pleomorphic rod forms in any of the 4 antigen areas. The size, appearance, and density of each field were compared with the Positive and Negative Control reactions. Patterns of reactivity different from the Positive Controls were considered non-specific.

Primary (initial) infection is characterized by a prompt rise in both IgG and IgM class antibody by IFA. IgM levels peak approximately 3 weeks post onset of symptoms and remain detectable for 2-3 months. IgG class antibody peaks in 7-12 weeks, but declines much more slowly than IgM antibody levels and remains elevated for approximately 12 months.

If the slides show positive at 1:64 or greater it suggests recent infection and if negative at 1:64 it is reported as negative.

This assay is not intended to determine strain-specificity of antibody reactivity but to improve test sensitivity by using 4 different strains.

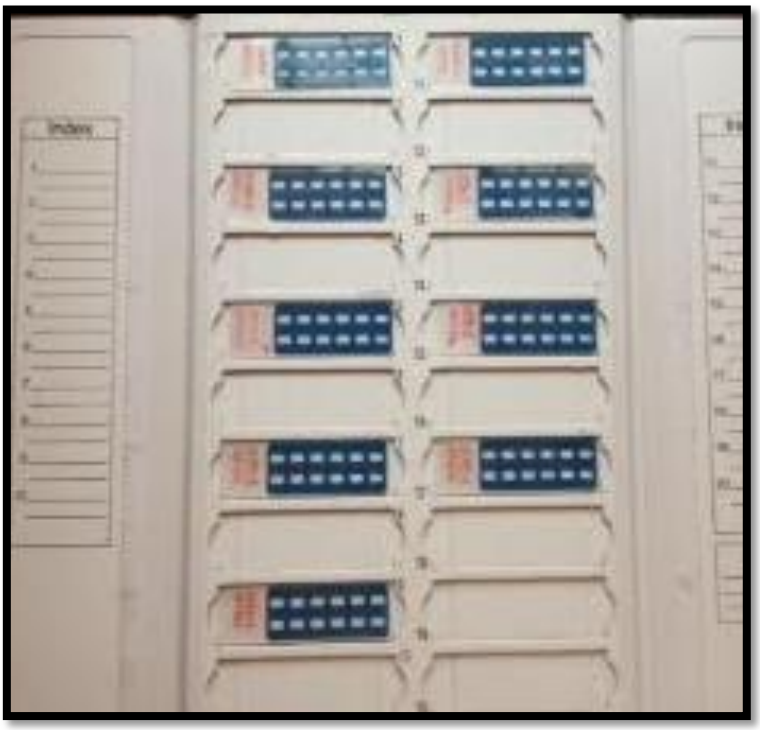


Fig4.1 IFA Slides in slide tray

Fig 4.2 Single IFA slide with 12 wells each well containing 4 acetone fixed strains

Fig 4.3 Fluorescent Microscope which was used for reading the IFA slides

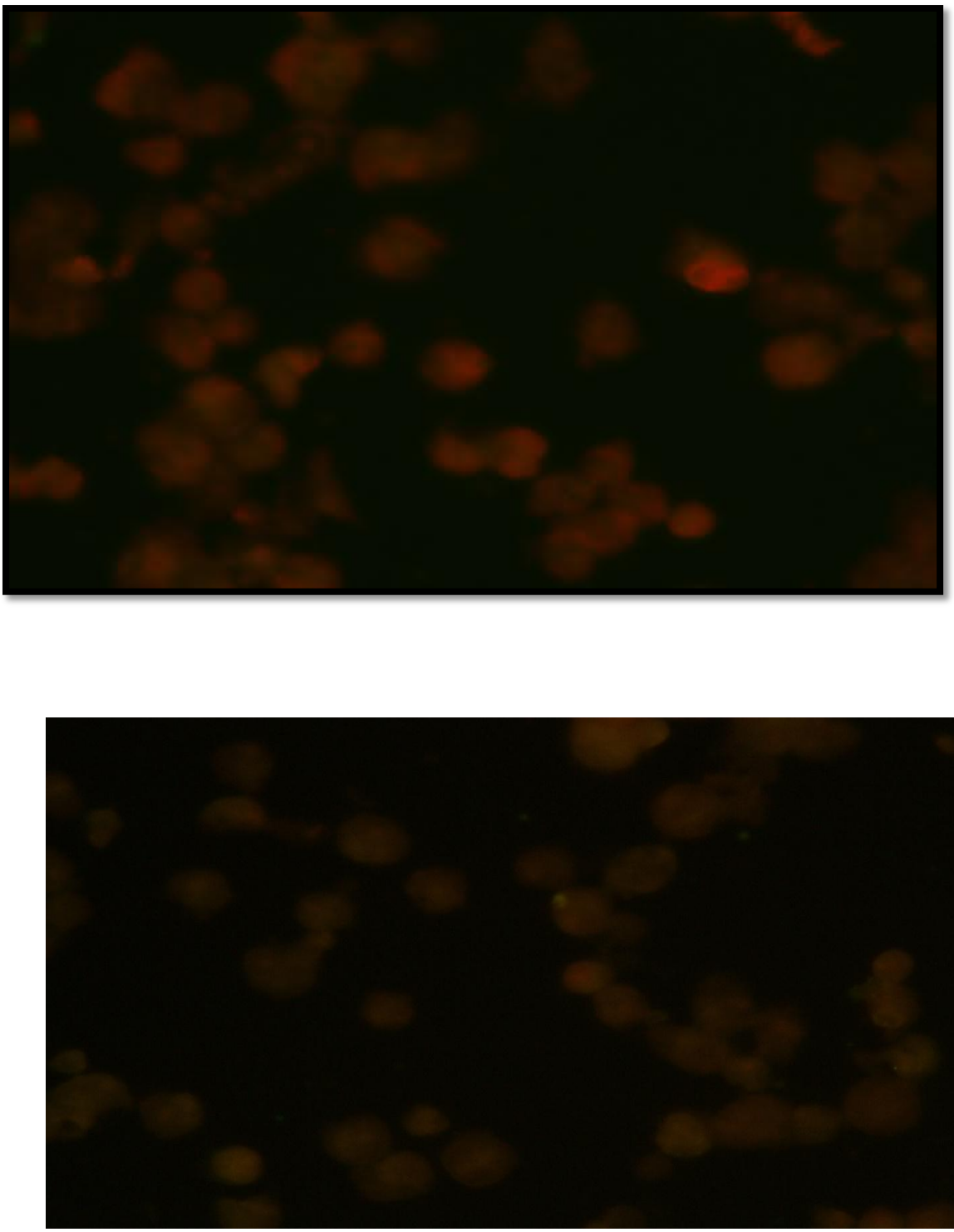


Fig 6.4. IFA slide well as seen under 400X magnification showing negative reaction. The cells L929 in which the strains have been propagated are seen stained red.

.

5. Results

During the study period from October 2012 to August 2014, a total of 633 cases of suspected scrub typhus presented to our hospital. Out of these, 95 serum samples were positive for scrub typhus either by Weil Felix or by ELISA (IgM antibody to O.tsutsugamushi). From these positive cases the following observations were made.

The patients predominantly presented with the complaints of fever half of which were associated with chills and rigors and the duration of fever was on an average for 10days. Other symptoms seen in decreasing order of frequency were headache, vomiting, cough, myalgia, breathlessness, loose stools, reduced urine output, abdominal pain, nausea, joint pain, eschar and constipation. Eschar was seen and documented in six of the cases. (Fig 5.1)

It was observed that of the 95 cases, males constituted 50.5 % and females constituted 49.4%. (Fig 5.2). It was also observed that about 50 % of these patients were those involved in agriculture.

The age distribution was seen as follows in the descending order. The highest numbers of cases were seen in the age group of 41 – 50yrs with 25 cases (26%). This was followed by age group 31 – 40 yrs with 18 cases (18.9%). The age groups 21 – 30 yrs and 51 – 60 yrs had 15 cases each (15.7%). The age group 61 – 70 had 9 cases (9.4%)

and 11 – 20 yrs had 8 cases (8.4%). Lesser number of cases was seen in the age group 2 – 10 with 3 cases (3.1%) and the least number of cases were in the age groups 71 – 80 yrs and 81 – 90 yrs with 1 case each (1.05%). No cases were observed in ages less than 2 and more than 85yrs. (Fig 5.3)

As for the season variation it was observed from the period of September 2012 to August 2014. A total of 41 cases were observed in the months of Sept to November, 40 cases from December to February, 15 cases from March to May and 20 cases from June to August. (Fig 5.4)

With respect to association of scrub typhus with other serologically diagnosable causes of fever with similar presentations, it was observed that of the 95 cases, 63 of them suffered from scrub typhus alone, 20 cases were Dengue and Scrub typhus positive, six cases were Widal and scrub typhus positive, two cases were Malaria and Scrub typhus positive, one was Infectious mononucleosis and Scrub typhus positive and three cases had more than two infectious test positive.(Fig 5.5)

The platelet count was found to be below 150 x 10^3in 30 (47.6%) out of 63 of the patients suffering from scrub typhus alone, in

17(85%) out of 20 of the patients with dengue and scrub typhus, in four out of the six patients with scrub typhus and widal positive and in two out of the two patients with scrub typhus and malaria. The patient who had infectious mononucleosis had normal platelet levels. Of the three patients who had more than two infectious diseases, one had levels less than 1.5 x 10^3, one had normal levels and one patient's platelet level was not available. (Table 5.1)

It was observed that out of the 95 cases 64 cases were documented to have been administered doxycycline and 9 cases were administered azithromycin. The remaining 21 cases data was unavailable.

Geographical mapping of the positive cases was also done. Coimbatore had 38 cases, Erode 17, Niligiris nine, Dindigul three, salem one, Namakal two and trichy one case.(Fig 5.6)

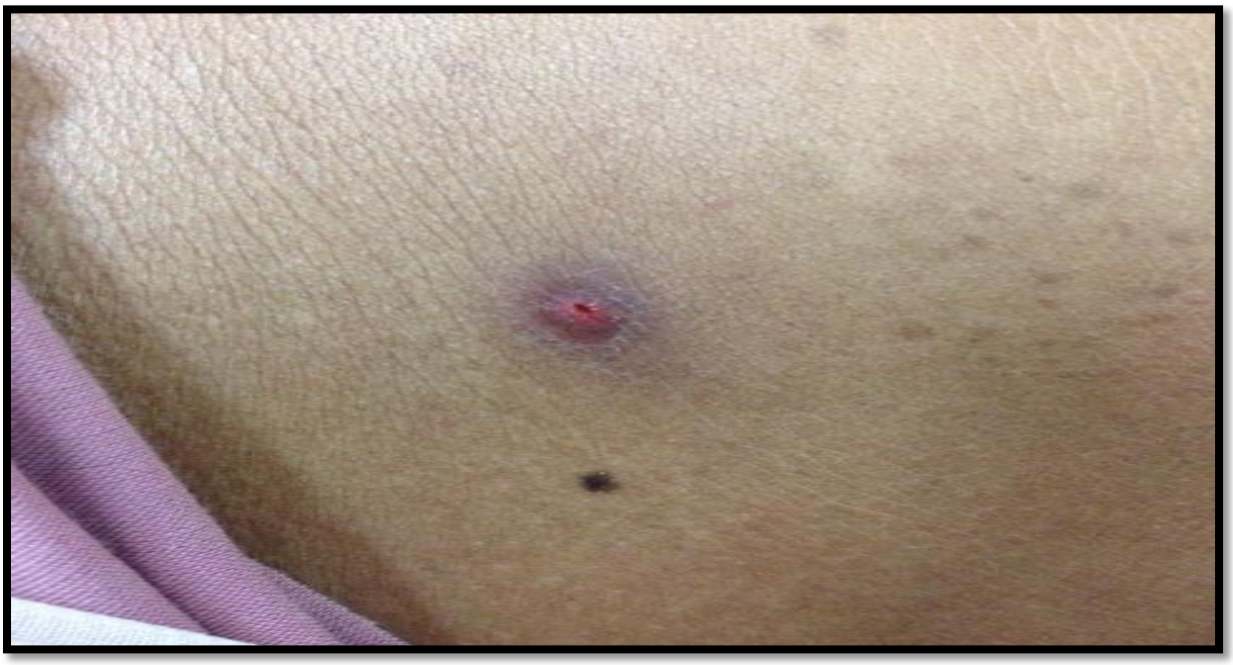


Fig 5.1: Picture of eschar seen over the lower right rib

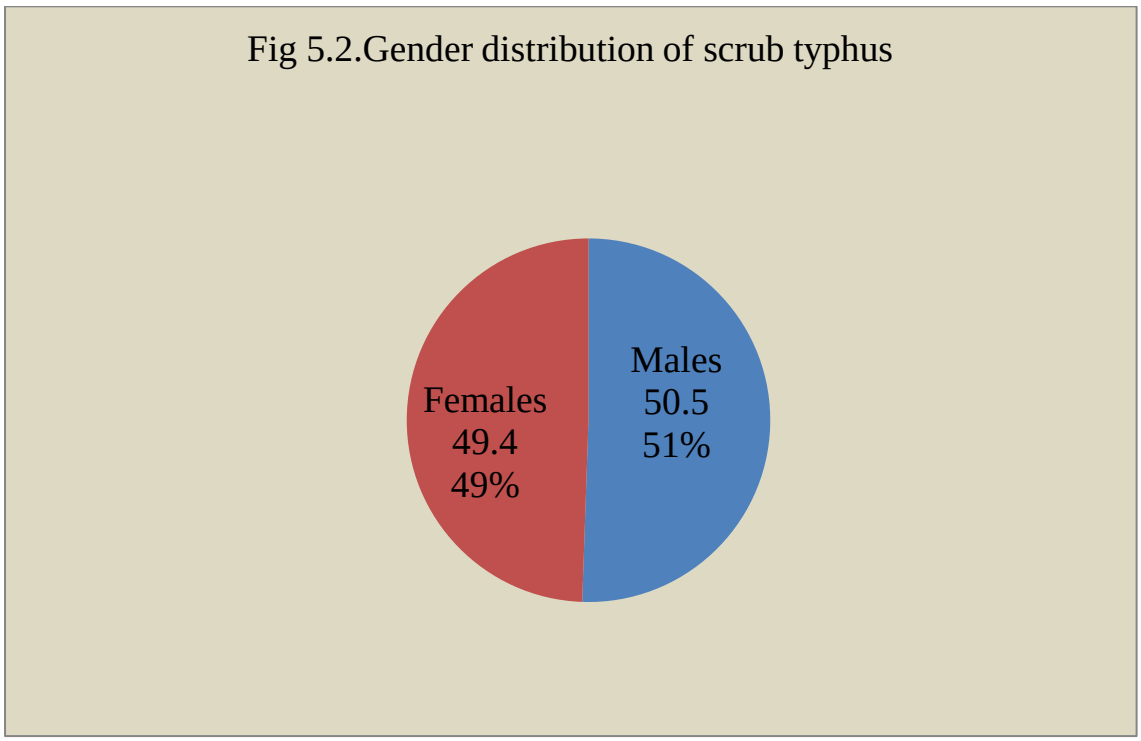


Fig 5.3 Age group distribution of scrub typhus cases

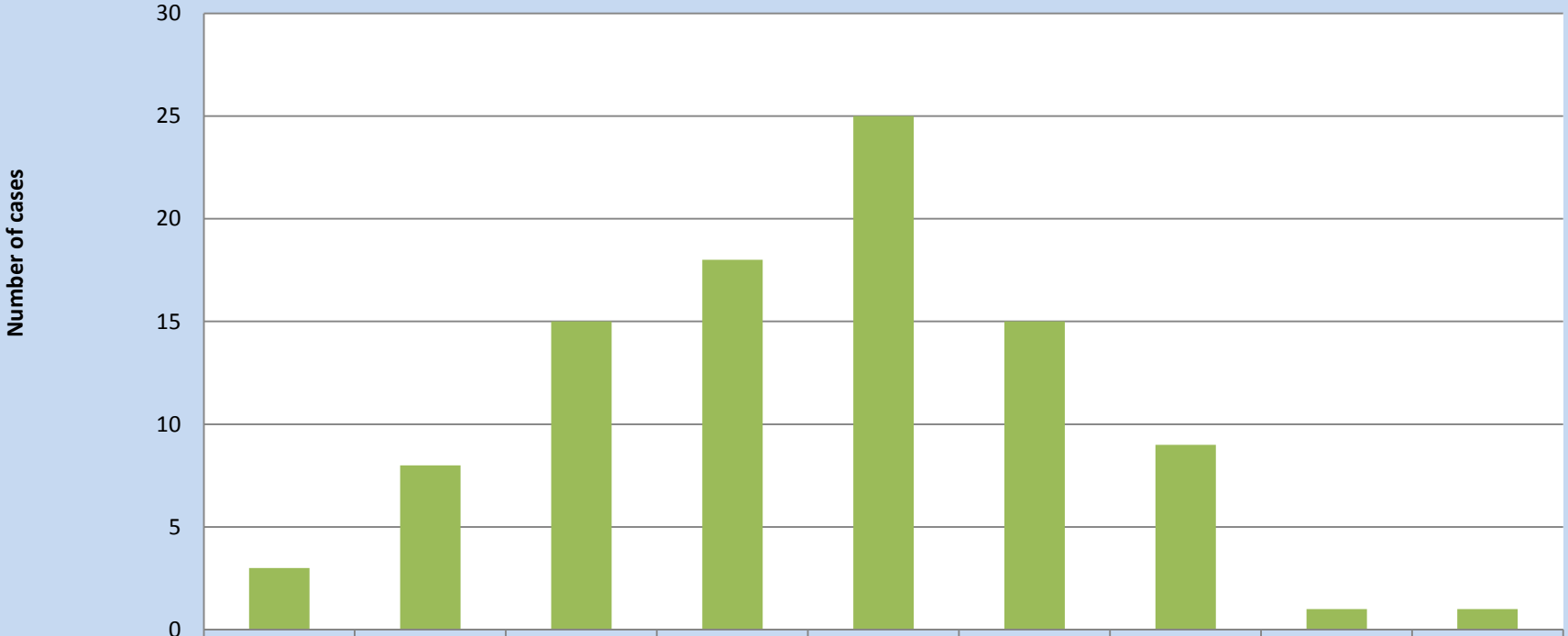
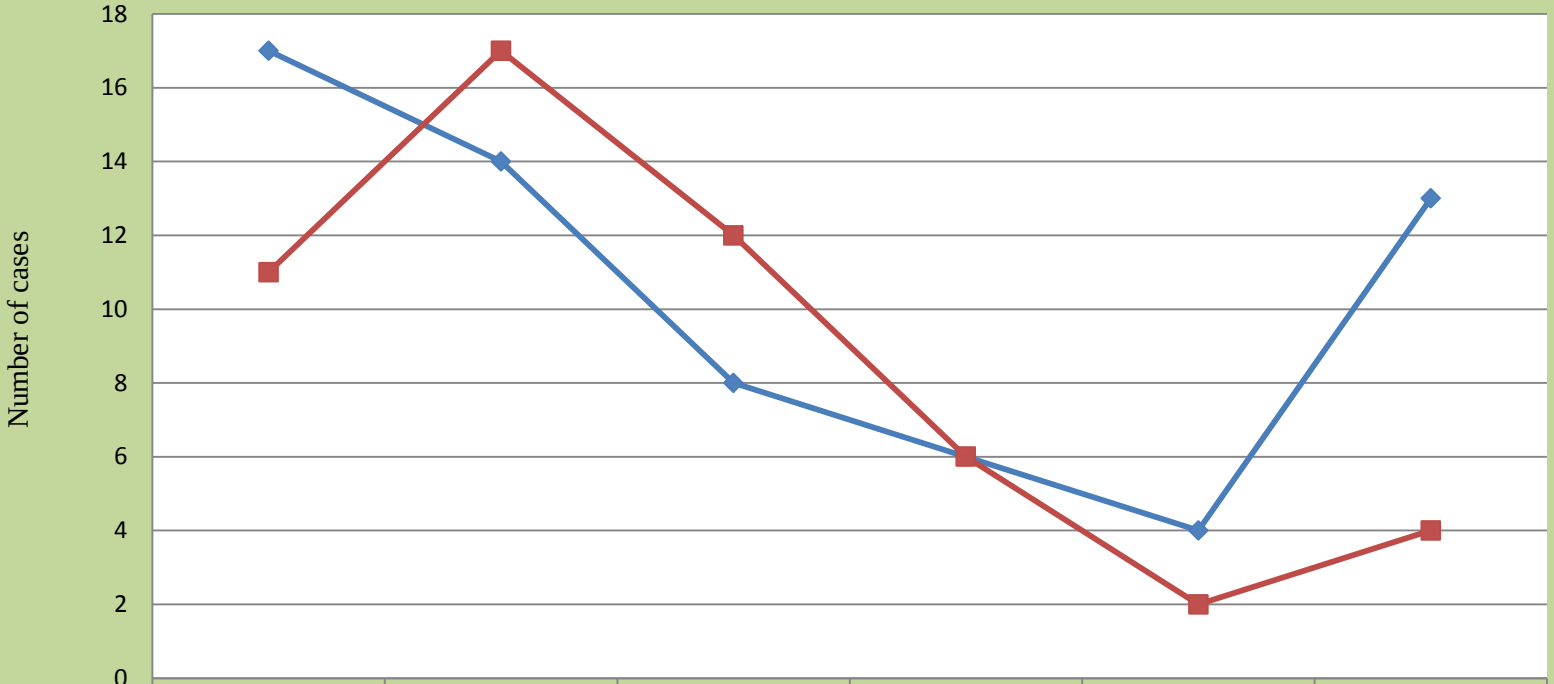
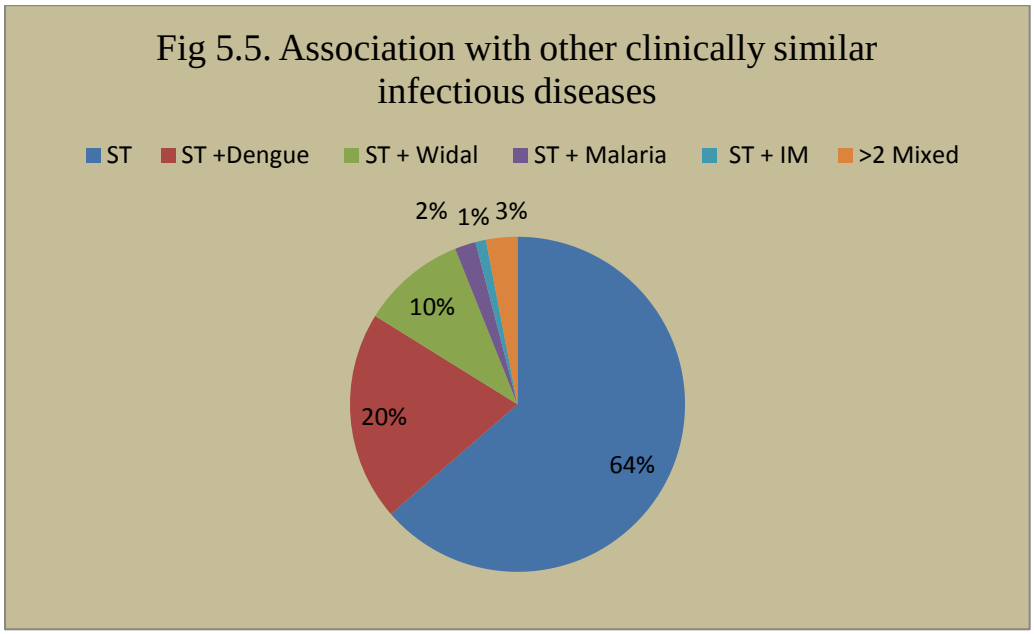


Fig 5.4 .Seasonal Variation of scrub typhus



	Sept - Oct	Nov - Dec	Jan - Feb	Mar - Apr	May - Jun	July - Aug
2012 - 2013	17	14	8	6	4	13
2013 - 2014	11	17	12	6	2	4



	NUMBER OF CASES	PLATELET <1.5 X 10^3	NORMAL PLATELET 1.5 – 4 X 10^3
SCRUB TYPHUS	**63**	**30**	**33**
ST+ DENGUE	**20**	**17**	**3**

Table 5.1. Platelet counts in Scrub typhus alone and Scrub typhus with Dengue.

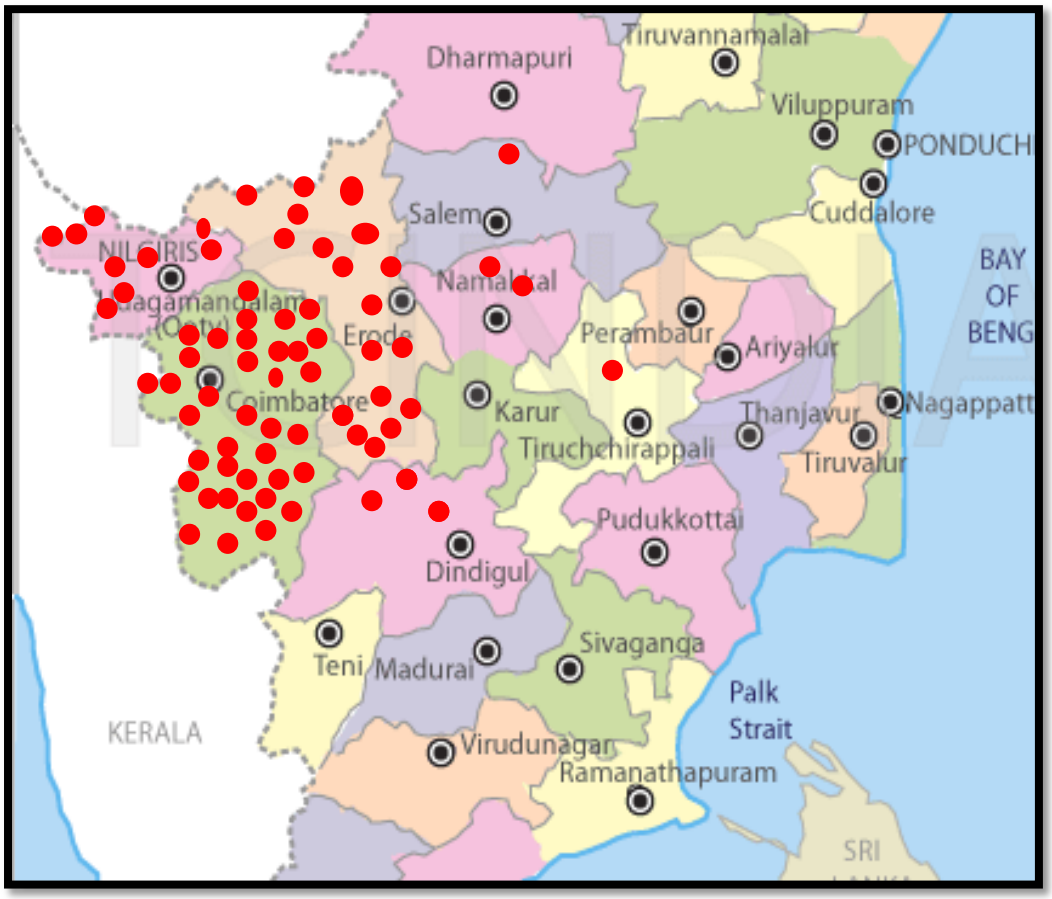


Fig 5.6 Map with positive cases of scrub typhus.

SCREENING TEST EVALUTION

Indirect Fluorescent Antibody assay which is the serological gold standard for Scrub typhus was done on serum samples of patients with suspected Scrub typhus. A total of 50 IFA negative and 50 IFA positive samples were selected. These samples were subjected to Weil Felix and ELISA (for detection of IgM antibody against Orientia tsutsugamushi) for comparison of results against IFA (Fig 6.5).

Of the 50 IFA positive cases, 20 were Weil Felix positive (at a titer of 1:160) and 48 ELISA positive, and 20 were both Weil Felix (at a titer of 1:160) and ELISA positive. Of the 50 IFA negative cases, 47 were Weil Felix (titer less than 1:160) negative and 44 were ELISA negative and 40 were both together negative.

The sensitivity of Weil Felix at a titer of 1:160 was found to be 40% and specificity 94%, Positive predictive value was 86.9% and negative predictive value 61.0%. The sensitivity of Weil Felix at a titer of 1:80 was found to be 54% and specificity 92%, Positive predictive value was 87% and negative predictive value 61%.The sensitivity of ELISA was found to be 96% and specificity 88%, Positive predictive value was 88.8% and negative predictive value 95.6 %. (Table 7.2 and 7.3)

	IFA +ve	IFA -ve
Weil Felix +ve at 1:160	20	3
Weil Felix –ve at 1:160	30	47

Table 5.2. Comparison of results of Weil Felix at a titer of 1:160 and IFA

	IFA +ve	IFA -ve
Weil Felix +ve at 1:80	27	4
Weil Felix –ve at 1:80	23	46

Table 5.3. Comparison of results of Weil Felix at a titer of 1:80 and IFA

	IFA +ve	IFA -ve
ELISA +ve	48	6
ELISA -ve	2	44

Table 5.4. Comparison of results of ELISA and IFA

	Weil Felix at 1:160	Weil Felix at 1:80	ELISA
Sensitivity	40%	54%	96%
Specificity	94%	92%	84%
PPV	86.9%	87%	88.8%
NPV	61.0%	61%	95.6%

Table 5.5. Parameters of Weil Felix and ELISA

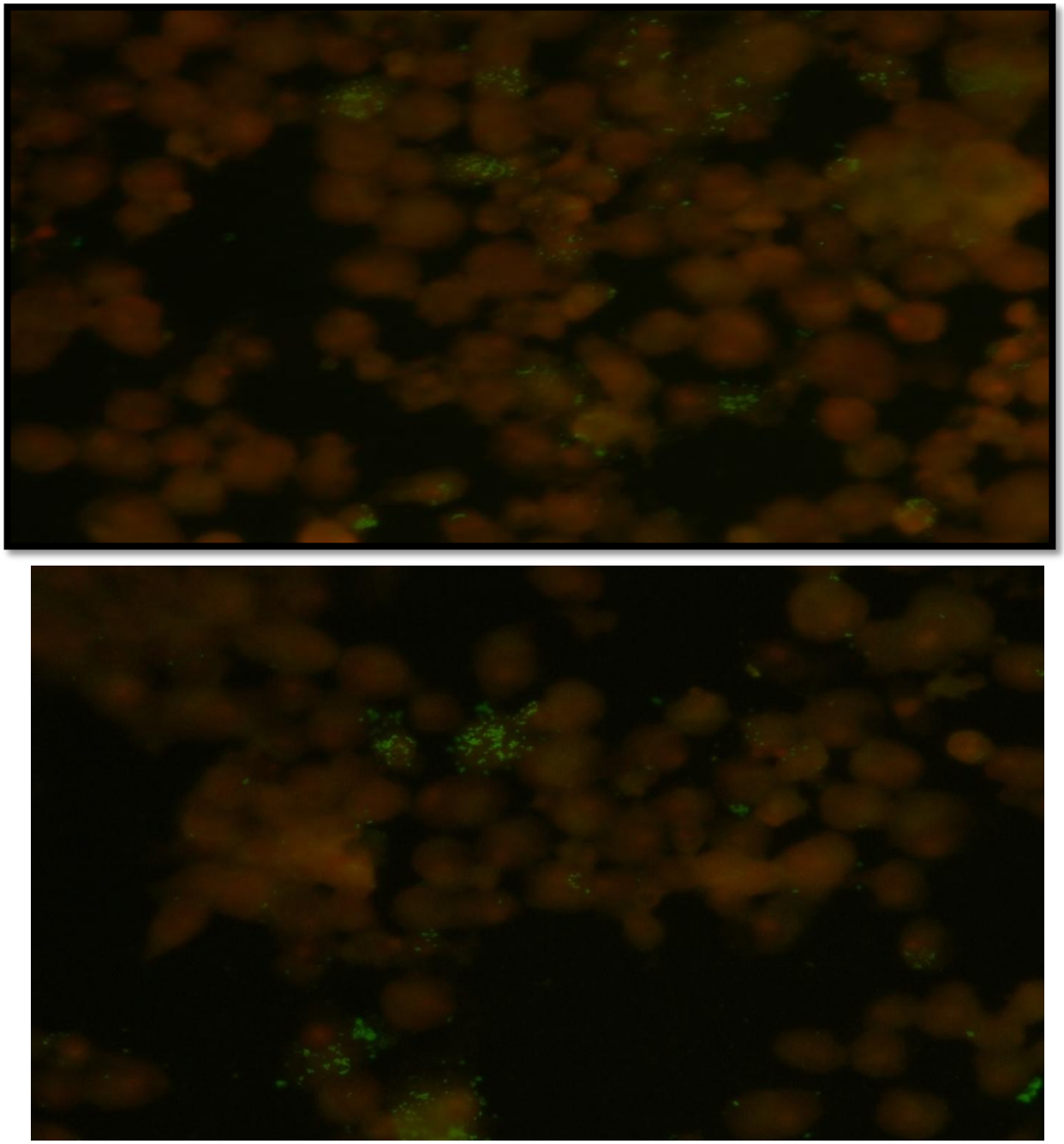


Fig 6.5 IFA slide well as seen under 400X magnification in Fluorescent microscope showing positive reaction. Small sharpe pleiomorphic fluorescent rods seen dispersed intracellularly and extracellularly.

6. Discussion

Scrub typhus disease is endemic to 13,000,000 km^2 area of the Asia – Pacific rim, extending from Afghanistan to China, Korea, the islands of the south western Pacific and northern Australia [5].Scrub typhus is a reemerging disease due to the increasing movement of people from rural to urban areas and deforestation activities. [9]

Scrub typhus is one of the causes of acute undifferentiated fever. Our study showed that of the 633 suspected cases of Scrub typhus from October 2012 to Aug 2014, 95 cases were positive by Weil Felix or ELISA constituting 15 % of the cases. In a study from Thailand, Scrub typhus accounted for 19.9% of the acute undifferentiated fevers [87].A study in Secunderabad showed a similar finding.[86]

Our study showed that the predominant clinical feature was fever with an average of 10 days followed by headache, vomiting, cough, myalgia, breathlessness, loose stools, reduced urine output, abdominal pain and nausea. The other features which we observed in our study were joint pain and constipation. These findings correlated with the other studies from Goa and Pondicherry [78, 79].

Eschar was observed in 6 out of the 95 patients (6.3%) in our study. The percentages of patients with scrub typhus having eschars are variable with different studies citing different results. The rate of the eschar depends on the investigators. A study from Korea showed that as much as 92.04% of the patients presented with eschar.[18] In Goa, one study showed that eschar was seen in 13.3% [78], from Pondicherry in 46%[79] .

It has been reported that the eschars were more frequently detectable in the fair skinned Japanese children than the dark skinned Thai children .It's also reported that in dark skinned patients the early eschar lesions were atypical and could be easily overlooked. A retrospective study on dark skinned Thai pediatric patients showed that only in 7% was the eschar detectable.[18] Another reason for variable reports could be because the presence of an eschar could be easily missed on routine physical examination and the vector bite is painless so the patients wouldn't notice it either [80]

In our study we observed an almost equal preponderance of scrub typhus in both genders. Out of the 95 cases, 48(50.5%) were

males and 47(49.4%) were females. Other studies showed variable reports with some showing male predominance and others showing female predominance. In a study from Andhra Pradesh, the males constituted 59.3% and females 40.7%[81] From Pondicherry the study showed out 50 patients 28 were females and 22 were males.[79]

Regarding the age distribution of Scrub typhus our study gave results with similar pattern to another study from KMC, Manipal.[83] Our study showed maximal incidence in the age group 41 – 50 yrs with decreasing incidence as one goes further from this peak to either side whereas the study from Manipal showed maximal peak in the age group 31 – 40 yrs.

About 50% of the patients were agricultural workers showing an increased risk among those involved in this occupation. Literature also reports Scrub typhus is generally seen among those whose occupation or recreational activities bring them in contact with the scrub vegetation. [71]

We observed for the seasonal variation from Sept 2012 to August 2014 and noticed an increased number of cases from September to February, which coincided with the cooler months of the year. It has been reported that outbreaks of scrub typhus in some areas

are seen more often in the cooler months [82]This could be due to the growth of secondary scrub vegetation (mite islands commonly seen in the post monsoon season from September to early months of next year) which is the habitat of the trombiculid mites.[16]. Some studies have shown the incidence of scrub typhus to be more in the rainy season[81].

Scrub Typhus presents as one of the causes of acute undifferentiated fever and this is made more complex by the presence of dual infections. Dual infections should be suspected when the patient presents with atypical clinical features of either disease or when patient responds poorly to treatment. [41]

In this study it was observed that of the 95 cases, 63 of them suffered from Scrub typhus alone and the remaining 32 cases suffered from mixed infections. Literature shows case reports of patients with Scrub typhus along with other infections but prospective studies for the incidence of the same were not found.[88,89]

Our study showed that the level of platelets less than 1,50,000/mm^3 was seen in 47.6% of those suffering from scrub typhus alone and in patient with scrub typhus and dengue, thrombocytopenia was seen in 85% of the patients. Thus correlating with study stating

that the platelet levels (<1, 40,000/mm^3) were found to be much lower in those suffering from Dengue as compared to scrub typhus infection. [84] Suputtamongkol et al from Thailand showed that thrombocytopenia was associated in 20.9% of the patients suffering from scrub typhus. [85]

In our study, the results of Weil Felix and ELISA were compared to the serological gold standard IFA. The sensitivity of Weil Felix OX K at a break point of 1:160 was found to be 40% and specificity 94%, positive predictive value was 86.9% and negative predictive value 61.0%.These results correlated well with other studies from Thailand and South India where both show Weil Felix OX K having a sensitivity of 30 – 40 % and specificity of 93.3%.

The ELISA containing r56 recombinant – antigen showed sensitivity to be 96% and specificity 88%, positive predictive value was 88.8% and negative predictive value 95.6 %. This was similar to the results stated in another study using the same assay and titer which showed sensitivity to be 97.5% and specificity to be 82.5%.

For the treatment of scrub typhus 67.3% of the cases were administered doxycycline, 9.4% was administered azithromycin and for the remaining cases the data was unavailable. One patient did not respond to doxycycline and combination therapy of azithromycin and rifampicin was given. The patient had responded better to the combination therapy but unfortunately succumbed to other complications.

7.Conclusions

Many of our finding have correlated well with other studies. The conclusions we have drawn based on this study are that, there is an occupational risk with those involved in agriculture, more in the age group of 41 – 50 yrs with an increasing incidence during the cooler months of the year.

Contrary to some studies we have observed good specificity with Weil Felix but it has a low sensitivity. Therefore if the results by Weil Felix are negative it does not rule out Scrub typhus and may require further testing. The good specificity we have observed could also be because of a low endemic titer in the local population. ELISA showed good sensitivity thus it is a preferable screening test though it is not very specific and may require confirmation with IFA when feasible.

Scrub typhus is a significant disease in this part of the country therefore it should be kept in mind as a possible diagnosis in undifferentiated fevers even though an eschar is not found. Scrub typhus was found to constitute 15 % of the acute undifferentiated fevers. This being a treatable disease further emphasizes the need for its timely and accurate diagnosis.

8.Summary

- Scrub typhus is one of the causes of acute undifferentiated fever. During the study period from October 2012 to August 2014, a total of 633 cases of suspected Scrub typhus presented to our hospital.

- Out of these, 95 serum samples were positive for scrub typhus either by Weil Felix or by ELISA (IgM antibody to Orientia tsutsugamushi).

- Predominant clinical feature was fever with an average of 10 days followed by headache, vomiting, cough, myalgia, breathlessness, loose stools, reduced urine output, abdominal pain and nausea.

- Eschar was observed in 6 out of the 95 patients (6.3%) in our study. The percentages of patients with Scrub typhus having eschars were variable with different studies citing different results.

- In our study we observed an almost equal incidence of Scrub typhus in both genders. Other studies showed variable reports with some showing male predominance and others showing female predominance.

- Maximal incidence of Scrub typhus according to our study was in the age group 41 – 50 yrs with decreasing incidence as one goes further from this peak to either side.

- Increased numbers of Scrub typhus cases were observed from September to February, which coincided with the cooler months of the year.

- Scrub Typhus diagnosis is made more complex by the presence of dual infections. Dual infections should be suspected when the patients present with atypical clinical features of either disease or when patient responds poorly to treatment.

- The platelet levels were low in Scrub typhus, but it was lower when the patient was infected with both Scrub typhus and Dengue infection.

- Main stay of Scrub typhus diagnosis remains serology. The gold standard for the diagnosis is Indirect Fluorescence Antibody assay.

- Both Weil Felix and ELISA tests were done and compared with IFA in 100 samples.

- The sensitivity of Weil Felix OX K at a break point of 1:160 was found to be 40% and specificity 94%, Positive predictive value was 86.9% and negative predictive value 61.0%

- The ELISA tests showed sensitivity to be 96% and specificity 88%, Positive predictive value was 88.8% and negative predictive value 95.6 %.

- All currently available serological methods have their limitations. There is an urgent need for newer diagnostic methods.

- The treatment given is Doxycycline or azithromycin. Most of our cases responded to these drugs except one case where combination therapy was attempted.

- Scrub typhus responds well to treatment and if not treated in time the patient can go in for complications emphasizing the need for early diagnosis and treatment.

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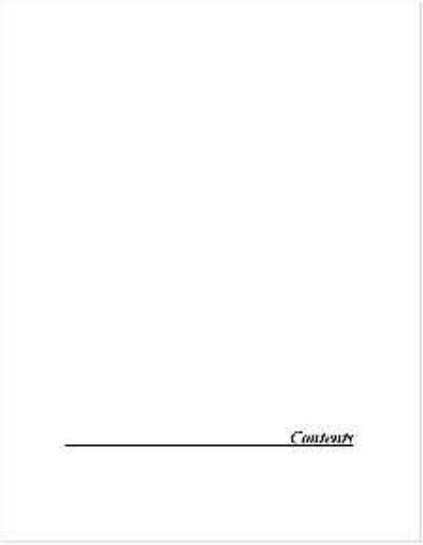

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