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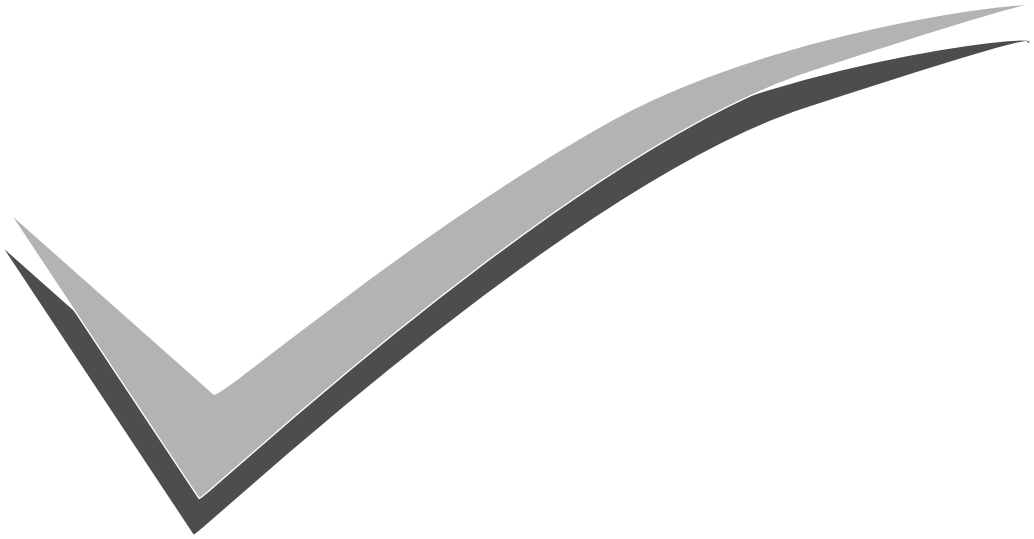

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ORIGINAL ARTICLE

Outbreak of Scrub Typhus in Andhra Pradesh – Experience at a Tertiary Care Hospital

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Abstract

Aim: To describe the clinical features, laboratory manifestations, complications in patients diagnosed with scrub typhus at a tertiary care hospital in south India.

Material and Methods: All cases of acute onset fever diagnosed to have scrub typhus August 2011 to December 2012 were analysed. Cases of scrub typhus confirmed by the weil felix test with a titre of 1 in 80 or more and a positive immunochromatography test were studied.

Results: 176 confirmed cases of scrub typhus were studied over a period of 18 months. Majority (96%) of patients are from rural background. Farmers constituted 60% of the patients. Most common symptoms were due to the involvement of respiratory tract in the form of cough in 94(53%) patients followed by breathlessness in 84 (47.7%). Signs of consolidation were seen in 80 (45.5%). Central nervous system involvement in the form of altered sensorium was seen in 43 (24.4%) and seizures in 11(6.3%) patients. Eshcar was seen in 23 (13%) patients. Transaminases were elevated in 153(86%) patients, serum alkaline phosphatase in 110 (62.5%) patients. Renal failure was seen in 49(27.8%) cases and respiratory failure was seen in 11 (6.2%). Eight (4.5%) patients died in our study.

Conclusion: Scrub typhus should be suspected in patients with rural background with fever and multi system involvement. The predominant symptoms were cough and breathlessness. Central nervous system abnormalities in the form of altered sensorium was seen in 43(24.4%). Most common laboratory abnormality noted in our patients with scrub typhus was elevated liver enzymes which were seen in 153 (86%) cases.

Introduction

Scrub typhus is caused by *Orientia tsutsugamushi* (*O.tsutsugamushi*) an obligatory intra-cellular gram negative bacterium. It is transmitted to humans by the bite of larval mites (chiggers) of *Leptotrombidium deliense*.[1] Scrub typhus, if undiagnosed or diagnosed late, or untreated, may prove fatal. The clinical manifestations of this disease range from sub-clinical disease to organ failure.[2,3] Fever is the most common feature of scrub typhus and due to lack of awareness among clinicians the condition is labelled as "fever of unknown origin".[4] scrub typhus has protean manifestations which can mimic conditions like pneumonia, meningoencephalitis, acute hepatitis, acute renal failure, loose motions and occasionally joint pains. Scrub typhus may occasionally present as fever of unknown origin and due to lack of awareness of the disease, clinicians spend a lot of time and resources during the work up. The clinical course of the disease and the prognosis vary depending on the character of the endemic strain.[5] Lack of access to specific laboratory tests is another problem in developing countries like India for the under diagnosis of several infectious diseases including scrub typhus. Recent reports from several parts of India, including South India, indicate that there is a resurgence

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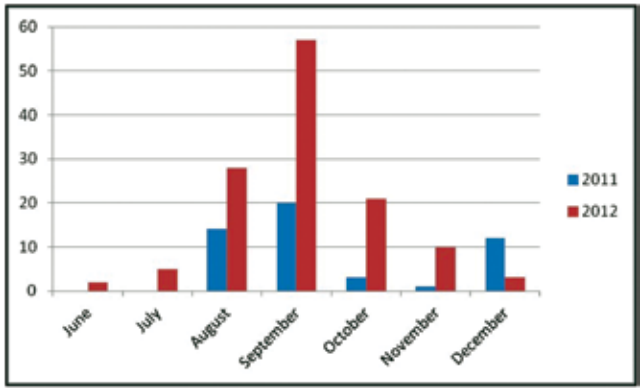

Fig. 1: Month wise distribution of cases in the study period 2011 and 2012

Fig. 2A: Maculopapular rash seen on the trunk

of scrub typhus.[2,6,7] There are very few publications on scrub typhus from the state of Andhra Pradesh.[8,9] We present our experience on clinical and laboratory features of patients with scrub typhus.

Material and Methods

This is a retrospective observational study of patients with scrub typhus who were admitted between August 2011 and December 2012. Data of clinical and laboratory features of patients aged more than 12 years, with fever, and confirmed diagnosis of scrub typhus was collected and analysed. Scrub typhus cases confirmed by the Weil Felix test with a titre of 1 in 80 and a positive immunochromatography test were included in the study. A positive Weil Felix OX – K titre more than 1 in 80 dilutions and a positive immunochromatography test were considered diagnostic of scrub typhus and were considered for the inclusion in the present study. Patients with other established causes of fever were excluded from this study.

Fig. 2B: Eschar on the anterior abdominal wall in a woman

Fig. 2C: Eschar on the right thigh of a farmer

Fig. 2D: Eschar on the trunk

The Weil Felix reagents used are Progen OX K Weil Felix reagent (Tulip Diagnostics, India). The other reagents are Progen OX 2 and Progen OX 19. The test was performed on an agglutination slide with the Weil Felix reagents by adding one drop (50 ul) of serum and one drop each of OX K, OX 2 and OX 19 reagents in separate circles of the card. It was mixed well with a mixing stick and observed for a visual agglutination after 1 minute. If it was positive, doubling dilutions of the serum was prepared and the test repeated. The highest dilution in which the agglutination visible was taken as the titre of the test. The dilutions included were:1 : 80, 1 : 160, 1 : 320, and 1 : 640. Any sera sample tested positive for agglutination with positive with OX K reagent, was retested by the scrub typhus immunochromatography test (ICT) (Standard Diagnostics, Seoul, South Korea) card test. The ICT

Table 1 : Showing baseline Characteristics of the patients

Characteristics of the patient	Number of patients (%)
Males	105(59.7%)
Females	71(40.3%)
Rural background	170(96.6%)
Urban but visited rural area	6(3.4%)
Occupation	
Farmers	104(59.1%)
Housewives	36(20.5%)
Skilled workers, Students, Businessmen	36(20.5%)

Table 2 : Showing the symptoms and signs

Symptoms	Number of patients(%)
Fever Duration < 7 days	63(35.8%)
Fever Duration 7-14 days	73(41.5%)
Fever Duration > 14 days	40(22.7%)
Cough	94(53.4%)
Breathlessness	84(47.7%)
Jaundice	40(22.7%)
Diarrhoea	28(15.9%)
Seizures	11(6.3%)
Status Epilepticus	1(0.5%)
Joint pains	5(2.8%)
Hepatosplenomegaly	51(28.9%)
Altered Mental Status	43(24.4%)
Eschar	23(13.1%)
Crepitations in lungs	80(45.5%)

consists of a filter paper strip coated with the cultured somatic antigens of *O. tsutsugamushi* (Gilliam, Karp and Kato strains). Ten microlitres of serum was added into the test well of the ICT device followed by 4 drops of buffer solution. A positive test was indicated by the development of a coloured band near the well of the device. An internal positive control was added for confirming the test result which was kept away from the test well. If found positive by the ICT as well, the test serum sample was diluted in doubling dilutions and ICT was repeated. The highest dilution showing a colour band in the test well was taken as the titre.

Results

Study population was recruited after the start of the monsoon season in 2011 August to the end of October 2012. Patients with scrub typhus were identified on the basis of clinical features and serology. Data of 176 patients with scrub typhus were recorded from the case records and were analysed. Most of the cases are seen in months of monsoon and post monsoon period i.e., August, September and October (figure1).

The baseline characteristics of the study patients are shown in Table1. Mean age of the study population is 41 years (±16). There were 105 (59.7%) males and 71(40.3%) females in our study. Most of the patients are from rural background with history of working in

Table 3 : Showing the Laboratory abnormalities

Investigation	Number of patients (%)
Mean Haemoglobin	11.1g/dl
Leucopenia	18(10.2%)
Leucocytosis	42(23.9%)
Platelets < 1 lakh	53(30.1%)
Elevated Serum Aspartate transaminase	153(86.9%)
Elevated Serum Alanine transaminase	136(77.3%)
Elevated Serum Creatinine	49(27.8%)
Elevated Serum Alkaline Phosphatase	110(62.5%)
Infiltrates on chest radiograph	46(26.1%)
Bradycardia	6(3.4%)

open fields. One hundred and seventy (96.6%) patients are from the rural part of Andhra Pradesh and the rest of the patients, i.e. 6 (3.4%) patients visited rural parts of the state in the recent past.

The clinical features of the patients are shown in Table 2. All patients presented with fever and the average duration of fever was 11.8 days (range 2 to 30 days). Majority of patients i.e. 73(41.5%) patients presented with fever of 7-14 days duration. Forty (22.7%) patients presented to us as prolonged pyrexia as fever was persisting beyond 14 days. Most common symptom was cough (53.4%). The next most common symptom was breathlessness, which was present in 84 (47.7%) patients. Loose motions were seen in 28(15.9%) patients. Seizures were present in 11(6.3%) patients and one patient presented with status epilepticus. Joint pains were seen in 5(2.8%) patients.

The most common abnormality on examination seen was lung involvement in the form of crepitations in 80(45.5%) patients. Twenty three (13.1%) patients had altered mental status. Eschar was seen in 23 (13.1%) patients in our series. Maculopapular rash was seen in 10 (5.7%) patients. The other signs of organ involvement observed were hepatosplenomegaly in 51 (28.9%) patients.

Laboratory features are shown in Table 3. Mean haemoglobin was 11.1g/dl (± SD±2.3). Leucopenia (TLC< 4000/cmm) was seen in 18(10.2%) patients. Leucocytosis was seen in 42(23.9%) patients. Thrombocytopenia (platelet count < 100000/cmm) was seen in 53(30.1%) patients. Elevation of serum creatinine i.e. 1.6 mg/dl was seen in 49(27.8%) patients. Elevation in serum aspartate transaminase (AST) was noted in 153(86.9%) and serum alanine transaminase (ALT) in 136 (77.3%) patients. Cardiac conduction abnormalities in the form of relative bradycardia (corresponding to temperature record) were seen on electrocardiogram in 6 (3.4%) patients. Dialysis was needed in 5 (2.8%) patients. Ventilatory support was needed in 8(4.5%) patients.

Average duration of hospital stay was 7.2 days (SD: ± 3.95). Duration of hospital stay of less than 7 days was noted in 121(68.8%) patients. Forty six (26.1%)

patients had a hospital stay of 8-14 days. Nine (5.1%) patients stayed for more than 14 days in the hospital. All patients were treated with doxycycline orally and supportive care. Out of 176 patients 168 (95.5%) recovered and eight (4.5%) patients died. One (0.5%) patient left against medical advice.

Discussion

It is important to rapidly delineate the cause of fever in regions where several infections like dengue fever, malaria, scrub typhus, and community-acquired pneumonia are common. Finding the exact aetiology is important as treatment is different for each disease and unnecessary use of antimicrobial agents can be avoided. In India, epidemics of scrub typhus have been reported from north, east and south India [5,6,7,10] but there are not many case reports of scrub typhus from the state of Andhra Pradesh till recently. We have observed a sudden outbreak of scrub typhus since the year 2011 from this part of the country.

O.tsutsugamushi, an obligate intracellular bacterium transmitted to humans by the bite of larval mites (chiggers) of *Leptotrombidium deliense*.[1] These larval mites usually feed on the wild rats of the subgenus Rattus. The organism is maintained by transovarian transmission in mites. There are several serotypes of *O.tsutsugamushi* and infections with one-serotype gives only transient cross immunity to another.[7] Man is accidentally infected when he encroaches the mite-infested areas with secondary scrub growth, which grows after the clearance of primary forest. The basic pathologic changes are focal vasculitis and perivasculitis of the small blood vessels in the involved organs, arising from multiplication of the organisms in the endothelial cells lining the small blood vessels.[11,12-14]

In the present study most of the cases were seen during the months of July to November. Scrub vegetation, optimum amount of monthly rainfall, and soil bound moisture are important factors responsible for disease transmission.[15] Consequently, an increase in incidence of cases is seen in the rainy season.[16] One more reason for increased incidence during the months of August to October is that, farmers are involved in the harvesting activity in the fields, where they are exposed to the bites of larval mites.[10] However, there are descriptions of scrub typhus outbreaks in cooler seasons also.[16] This is possibly due to the growth of secondary scrub vegetation, which is the habitat for trombiculid mites (mite islands in the immediate post monsoon period i.e. September to early months of the next year).[1]

As described in literature the disease is common in farmers, persons rearing domestic animals and those living close to bushes and woodpiles.[17] Farm work and related activities were noted in 60% in our study and it is comparable to other studies in the literature.[18,19] Almost all the patients were from rural background in our study. The mean age of our patients is 41 years and majority were men. Age and sex can occasionally influence the incidence of scrub typhus mainly due to the exposure to outdoor activities in the younger adults. Whether occupational or recreational, more common in 21-50 years and those involved in outdoor activities.[20]

The classic case description includes an eschar at the site of chigger feeding, regional lymphadenopathy, and a maculopapular rash.[1,21] An eschar at the wound site is the single most useful diagnostic clue and it is very important to perform a thorough physical examination to look for eschar and signs to exclude other causes of fever. Though eschar was pathognomonic of scrub typhus, it was noted only in 23 (13.1%) of our patients. Similar number of eschars reported in other Indian studies by Mathai *et al* (2003) and Vivekananda *et al* (2010). The reason for the less number of eshcar in Indian studies may be due to the high skin colour of the population and due to variation of serotypes among different regions.[6] Maculopapular rash was seen in 10 (5.7%) patients in our series and is comparable to other Indian studies.[7,15]

Most of our patients with scrub typhus presented with non-specific symptoms of gastrointestinal and respiratory tract involvement mimicking viral fever. One of the clinical differentiating features in scrub typhus from other viral illnesses like dengue is the duration of fever. Average duration of fever in our study group was 11.8 days (ranging from 2 to 30 days). Forty (22.7%) patients presented with prolonged pyrexia i.e., fever more than 2 weeks duration.

Scrub typhus involves multiple organs including the lung, heart, central nervous system (CNS), and is characterised by focal vasculitis or perivasculitis. Table 4 shows the clinical features of our study in comparison with other series. Such microangiopathies may also involve the kidney (acute renal failure), gastrointestinal tract (gastrointestinal bleeding), liver (hepatic dysfunction and hepatomegaly), spleen (splenomegaly), and lymph node (lymphadenopathy).[12]

Respiratory tract involvement is a common manifestation of scrub typhus and clinicians need to differentiate it from community-acquired pneumonia caused by the usual organisms like streptococcus species. Cough and breathlessness were present in 94 (53.4%) and 84 (47-7%) patients respectively in our series. Eighty (45.5%) patients had signs of consolidation on clinical examination. Respiratory failure is a common complication of scrub typhus and was reported in 11% of cases in one large series.[22] In our series, 11(6%) patients developed respiratory

Table 4 : Showing Comparison of Clinical features of scrub typhus from various studies

Clinical Features	Mathai et al 2003	Mahajan et al 2006	Kun-Ming Wu et al 2007	Vivekanandan et al 2008	Kedareshwar et al 2010	Present study 2013
Place of study	Vellore	Shimla	Taiwan	Pondicherry	Goa	Andhra Pradesh
No. of subjects studied	28	21	136	50	15	176
Fever	27(100%)	21(100%)	134(98.5%)	50(100%)	15(100%)	176(100%)
Myalgia	14(52%)	38%		19(38%)	12(80%)	
Altered mental status	5(19%)	24%		10(20%)	1(6.7%)	23(13.1%)
Headache	9(33%)		85(62.5%)	20(52%)		92(52.3%)
Cough	12(44%)		71(52.5%)	20(40%)	7(46.7%)	94(53.4%)
Breathlessness				13(26%)	9(60%)	84(47.7%)
Nausea, vomiting	13(48%)	43%		29(58%)	15(100%)	
Pain abdomen		29%	42(30.9%)	10(20%)	7(46.7%)	
Diarrhoea				8(16%)	4(26.7%)	28(15.9%)
Hepatosplenomegaly		43%	35(34.7%)	24(48%)	13(86.7%)	51(28.9%)
BP < 90 mmhg				8(16%)	7(46.7%)	
Eschar	1(4%)	10%	82(60.3%)	23(46%)	2(13.3%)	23(13.1%)
Leucopenia < 4000/cmm				1(2%)		42(23.9%)
Leucocytosis > 11000/cmm	14(54%)		39(29.8%)	15(30%)	10(66.7%)	18(10.2%)
Thrombocytopenia < 100000/cmm	9(43%)			5(10.8%)	4(26.7%)	53(30.1%)
Hepatitis	22(88%)		109(85.8%)	47(95.9%)	12(80%)	153(86.9%)
Renal failure	8(37%)		9(6.8%)	6(12%)	5(33.3%)	49(27.8%)
Mortality	3(11%)		215%)	1(2%)	533.3%)	8(4.5%)

failure out of which 8 patients required ventilator support and 3 patients needed high flow oxygen support. Chest radiograph abnormalities in the form of reticulonodular opacities, air space consolidation, peribronchial infiltration, pulmonary congestion, pulmonary oedema, acute respiratory distress syndrome (ARDS) and pleural effusion were known to occur in scrub typhus.[22,23] We did not measure the chest radiograph abnormalities in our patients in this study.

Gastrointestinal system symptoms in the form of vomitings and loose motions are common presenting features of scrub typhus and are reported in many studies (Table 5). Loose stools were seen in 28(15.9%) patients in our series. Clinician should suspect scrub typhus in a case of fever and diarrhoea if accompanied by symptoms of respiratory or central nervous system symptoms in an endemic area and it helps in differentiating from infective diarrhoea. Hepatosplenomegaly was seen in 51(28.9%) patients of our series. Among the gastrointestinal manifestations, elevated hepatic transaminases are a striking feature in scrub typhus that physicians need to pay attention in an endemic area. We have observed an elevation in transaminases in which ALT was found to be more than AST similar to the reports found in literature.[4] Apart from transaminases, elevated serum alkaline phosphatase and serum bilirubin were seen in 110 (62.5%) and 68(38.6%) patients respectively in our study in comparison to other studies.[7,15,24]

Renal failure was the next complication after hepatitis in our series. Elevation of serum creatinine > 1.6 mg/dl was seen in 49(27.8%) patients in which 5(2.8%) patients required dialysis. Renal failure was seen in 13- 37%[7,15] from two different studies from India.

Scrub typhus, as the name suggests is characterised by fever with altered sensorium. CNS involvement ranges from aseptic meningitis to frank meningoencephalitis.[25] The pathologic changes in the brain are predominantly vascular in nature and actual tissue destruction is rare and they are potentially reversible despite widespread lesions.[22] As reported in other series in literature, 33 (18.8%) of our patients had drowsiness on examination during hospital stay. Seizures were present in 11(6.3%) and one patient presented with status epilepticus. All patients including the one with status epilepticus responded to treatment with doxycycline.

The existence of myocarditis in scrub typhus is easily ignored, because the symptoms of myocardial involvement are usually subclinical and sometimes may lead to heart failure also.[26] We did not measure the myocarditis in our patients. Cardiac conduction abnormalities in the form of bradycardia were seen on electrocardiogram in 6 (3.4%) patients. and we observed bradycardia on ECG in 6(3.4%) of our patients. Other febrile illnesses with relative bradycardia include dengue fever, brucellosis, chlamydiosis, legionellosis and enteric fever.[27]

Another feature noted in our series was arthralgia in 8(4.5%) which was rarely reported in literature and only one study from India by Patil et al reported in 27% patients in their study from Karnataka.[28] Arthralgias are commonly described in other febrile illnesses like dengue fever and chikungunya fever and

need to be differentiated from each other as treatment is different for scrub typhus.

Complete blood counts and peripheral smear examination initially help in differentiating aetiology of fever, which is important in developing countries like India where there are limited resources. Among the laboratory abnormalities, most common haematological abnormality noted was thrombocytopenia in 53(30.1%) patients followed by leucocytosis in our series. Similar observation was made by other studies. This helps in clinching the diagnosis to scrub typhus. In complicated falciparum malaria there will be anaemia, leucopenia usually and thrombocytopenia occasionally. In dengue fever there will be haemoconcentration, leucopenia and thrombocytopenia.

We utilised Weil-felix test and immunochromatography to diagnose cases of scrub typhus in our patients. Indirect immunofluorescence test is the gold standard diagnostic test for scrub typhus.[29] Though Weil-Felix agglutination test is not a very sensitive test, it has high specificity and positive predictive value.[30] A good correlation between the results of Weil Felix test and the detection of IgM antibodies by an Immunofluorescence assay has been observed.[7,31] Due to lack of availability of definitive tests in India Weil felix can be a useful tool when used and interpreted in correct clinical context.[30,32]

Antibiotics of the tetracycline class (doxycycline in particular) have a high degree of efficacy and low toxicity in treating rickettsial infections, even in children and pregnant women. The treatment of choice for scrub typhus infection is doxycycline 100 mg per dose administered twice daily (orally or intravenously) for adults or 2.2 mg per Kg for children less than 45.5 Kg.[33] This treatment should be started empirically as soon as diagnosis is suspected. The optimal duration of treatment has not been established, but current recommendation suggests at least 3-7days for life threatening cases to a maximum of 15 days for severe or complicated disease. Alternatively chloramphenicol (500 mg 4 times a day orally for 7 days in adults or 150 mg per kg per day for 5 days in children) in endemic areas has been proven effective in treating scrub typhus and preventing relapse.[33] Rifampicin or azithromycin are effective in doxycycline resistant strains of scrub typhus.

Outcome of the patients admitted with scrub typhus was studied. All our patients are treated with doxycycline and supportive care. Doxycycline was given for 7 days in mild disease and 14 days for patients with multiorgan disease. Out of 176 patients 168 (95.5%) recovered, one (0.5%) patient left against medical advice and eight (4.5%) patients died. The main cause of death is ARDS and the mortality in our study is comparable to other Indian studies.[7,16,30] and a large study from Taiwan reported less (2-3%) mortality. The low mortality in Taiwan may be due to the endemicity of the disease and familiarity of the disease among the clinicians. The deaths in our series are possibly due to delayed diagnosis, late presentation and multiorgan dysfunction.

Conclusion

Scrub typhus clinically mimics infections like dengue viral infection, leptospirosis, malaria, pneumonia as all of them may be associated with sudden onset fever, mild hepatitis and thromocytopenia. Associated respiratory involvement, gastrointestinal symptoms, altered sensorium and hepatitis should prompt physicians for a diagnosis of scrub typhus. Majority of our patients with scrub typhus are from rural parts of Andhra Pradesh. Though eschar is pathognomonic of the disease, it was noted in 23 (13.1%) patients and its absence does not rule out scrub typhus. Respiratory symptoms are the most predominant symptoms while transaminases are the most predominant laboratory abnormality in our series. We conclude to say that in rural Andhra Pradesh patients who present with fever, respiratory symptoms and hepatitis scrub typhus should be suspected. As delay in treatment may lead to complications and higher mortality, empiric treatment with doxycycline or macrolides may be given in cases where scrub typhus is suspected and facilities for diagnosis are not available.

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Prevalence and Molecular Characterization of Scrub Typhus in Sub-Urban Regions of Vijayawada, Andhra Pradesh, India

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A B S T R A C T

Scrub typhus is one of the most under-diagnosed and under-reported febrile illnesses. Hence, a study was conducted to diagnose the scrub typhus by using Weil Felix test (WF), Immuno-Chromatographic Test (ICT) and nested PCR (N-PCR). The primer pairs encoding the 56-KDa antigen were used for confirmation of *Orientia tsutsugamushi*. Serotype-specific primers were used for identification of Gilliam, Karp, Kato, Kawasaki and Kuroki strains. Out of the 46 blood samples collected from suspected human patients, 13 were found serologically positive by both WF and ICT and 12 by N-PCR. All the 12 N-PCR positive samples were found positive for Karp strain. None of the rat blood samples were positive for *O. tsutsugamushi.*

Introduction

Rickettsial diseases are considered as some of the most covert emerging and re-emerging diseases and are being increasingly recognized. Among these, scrub typhus is the commonest occurring rickettsial infection in India. Scrub typhus is widespread in the Asia-Pacific region, known as the "*tsutsugamushi* triangle" (Chang, 1995). It is prevalent in many parts of India and outbreaks are reported during the cooler months of the year, usually June to November (Dumler and Siberry, 2007 and Mathai *et al.,* 2003). According to the World Health Organization, "Scrub typhus is probably one of the most under-diagnosed and under-reported febrile illnesses requiring hospitalisation in some regions" (WHO, 2006). Due to lack of awareness among clinicians the condition is labelled as "fever of unknown origin" (FUO) (Mahajan, 2005 and Walsh *et al.*, 2001).

Scrub typhus is an acute, febrile disease caused by *Orientia tsutsugamushi*. It is of zoonotic importance and the infection is transmitted through the bite of larval mites or "chiggers" belonging to the family Trombiculidae (*Leptotrombidium deliense*). The field rodent and vector mites act as reservoirs and between these two, the infection perpetuates in nature. A large

number of serotypes exist for *O. tsutsugamushi* out of which Gilliam, Karp, Kato and Kawasaki are commonly found (Kelly *et al.,* 2009).

Endemic foci are usually associated with specific habitats such as abandoned plantations, gardens or rice fields, overgrown forest clearings, shrubby fringes of fields and forests, river banks, grassy fields etc. where the mites naturally inhabit. Incidence of scrub typhus is higher among rural population (Sharma *et al.,* 2010) and the disease is seasonal in many parts of India, which correlates with the appearance and activity of mites (Manuj *et al.,* 2015).

Clinical symptoms of scrub typhus range from sub-clinical disease to multi-organ failure (Saah, 2000). An eschar is often seen in humans at the site of the chigger bite. Bites are often found on the groin, axillae, genitalia or neck (Lerdthusnee *et al.,* 2003). The illness begins with shaking chills, fever, severe headache, and myalgia. If untreated may lead to complications like leucopenia, abnormal liver function tests, pneumonia, dyspnoea, meningoencephalitis, acute hepatitis, acute renal failure, myocarditis, joint pains etc. (Yang *et al.,* 1995). Clinical signs and symptoms of scrub typhus in humans are largely nonspecific, and if it is not treated promptly and appropriately, it carries a high mortality rate as high as 30-45 percent with multiple organ dysfunction (Manuj *et al.,* 2015).

In India, many of the Scrub Typhus cases go unidentified due to limited availability of accurate diagnostic facilities. Many cases have been admitted into different corporate hospitals of Vijayawada with suspected symptoms of Scrub Typhus. So keeping this in view, the present study was undertaken to know the prevalence of Scrub Typhus by using WF test, ICT and N-PCR.

Materials and Methods

Standard control and primers

Standard DNA obtained from Dept. of Virus Research, Manipal University, Karnataka was used as positive control. Oligonucleotide primers were custom synthesized from M/s. Bioserve Biotechnologies Pvt. Ltd. (Hyderabad).

Sample collection

A total of 46 blood samples, collected from FUO patients with fever for >1wk were obtained from different hospitals of Vijayawada for diagnostic confirmation. We collected blood samples of 25 field rats trapped from different sub-urban areas of Vijayawada where the disease was prevalent. Two ml of blood samples were collected without anticoagulant, serum and blood clot were separated and preserved at –80°C until processing. All the human and rat blood samples were tested serologically by 2 methods- WF test and commercially available lateral-flow-format ICT. Further confirmation was done by using N-PCR.

ICT and WF tests

ICT is based on detection of IgM antibody against *O. tsutsugamushi.* (SD Bioline *tsutsugamushi* assay, Inc., Korea) and WF test is an agglutination test that detects cross reacting antibodies to *Proteus mirabilis* OX-K antigen (Progen OX-K antigen, Tulip Diagnostics (P) Ltd., India) (Raoult, 2009). The tests were conducted according to the manufacturer's instructions.

Nested PCR

All the serologically positive samples were subjected to N-PCR for confirmation of scrub typhus. DNA was extracted from the stored

blood clots using phenol-chloroform-isoamylalcohol method and subjected to N-PCR targeting the 56-KDa type-specific antigen (TSA) gene of *O. tsutsugamushi* using two sets of primers (Table 1). Primer set 1 amplified a 1003-1030 bp fragment which was purified by using QIAquick PCR purification kit (Qiagen, USA). The purified product is further used as template for N-PCR where primer set 2 amplified a product of 481-507 bp. The purified product was also used as template for strain-specific identification by using Kuroki, Karp, Kato, Gilliam and Kawasaki strain-specific primers with an amplification size of 407, 220, 230, 242, 407 and 523 bp respectively (Table 2). Forward is same for all the strains. PCR assays were optimized in 25µl reaction mixture containing 2µl of DNA template, 12.5µl of 2x PCR master mix (Go Taq Green Master Mix, Promega), 1µl each of forward and reverse primers (10pmol/µl) and the rest of the volume is made by adding nuclease free water, under standardized cycling conditions (Table 3).

Results and Discussion

Of the 46 suspected human blood samples, 13 (28.2%) were found serologically positive by both WF and ICT (Fig. 1) methods. The titer more than or equal to 1:160 was taken as positive for WF reaction. 1:160 titer was found in 2 patients, 1:320 in 3, 1:640 in 2, 1:1280 in 4 and 1:2560 in 2 patients.

Out of 13 serologically positive samples, 12 showed presence of *O. tsutsugamushi* specific 56KDa surface antigen by N-PCR. Further these samples were subjected to strain specific PCR amplification and all of them were found positive for Karp strain. The standard DNA which was used as positive control was found to be Kato strain (Fig. 2). All the rat sera and blood samples were found negative to *O. tsutsugamushi*. All the 13 positive human cases were reported to be suffering with fever, headache, myalgia and 46% (6/13) of them were presented with jaundice, 31% (4/13) with dyspnoea, 23% (3/13) with kidney failure, 15% (2/13) with thrombocytopenia and 15% (2/13) with hypotension. Eschar was seen in 4 patients out of 13 confirmed cases (Fig. 3).

All the 13 serologically positive patients belonged to rural areas and all of them were admitted into ICU with two or more of the above given complications. Most of the confirmed cases were suffering with jaundice followed by dyspnoea and kidney failure. Subbalaxmi *et al.,* (2014) reported that respiratory symptoms are the most predominant symptoms among the scrub patients. WF test showed titers ranging from 1:160 to 1:2560. Increase in titers was observed with increased duration of fever and associated complications. Usha *et al.,* (2015) reported titers ranging from 1:160 to 1:1280 by using WF test. Out of 13 serologically positive samples, 12 were found positive by N-PCR showing first PCR product at 1003-1030 bp and second product at 481-507 bp. All the N-PCR positive samples were found positive for Karp strain and Karp strain is one of the high virulence serotypes of *O. tsutsugamushi* (Groves and Osterman, 1978). Varghese *et al.,* (2013) reported prevalence of Karp and Kato strains in Vellore, Tamilnadu and Usha (2014) reported prevalence of Karp and Kawasaki strains in Andhra Pradesh. All the rat sera and blood samples were found negative for scrub typhus. Veena *et al.,* (2012) reported chigger index of 5.75 which was higher than the critical limit of 0.69/ rodent, but all the rodent serum (18) and impression tissue smears (20) were found to be negative for *Orientia tsutsugamushi*. Candaswamy *et al.,* (2016) reported a chigger index of 41.1/animal where 28 out of 50 samples were positive by WF test and only 2 showed presence of *O. tsutsugamushi* specific $GRoE_1$ gene by PCR.

Fig.1 ICT showing test positive for scrub typhus

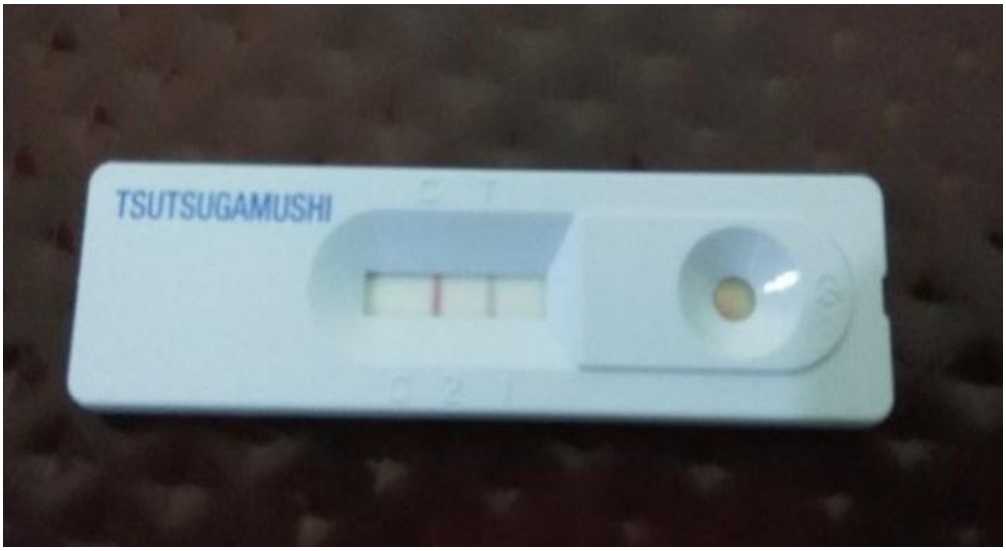
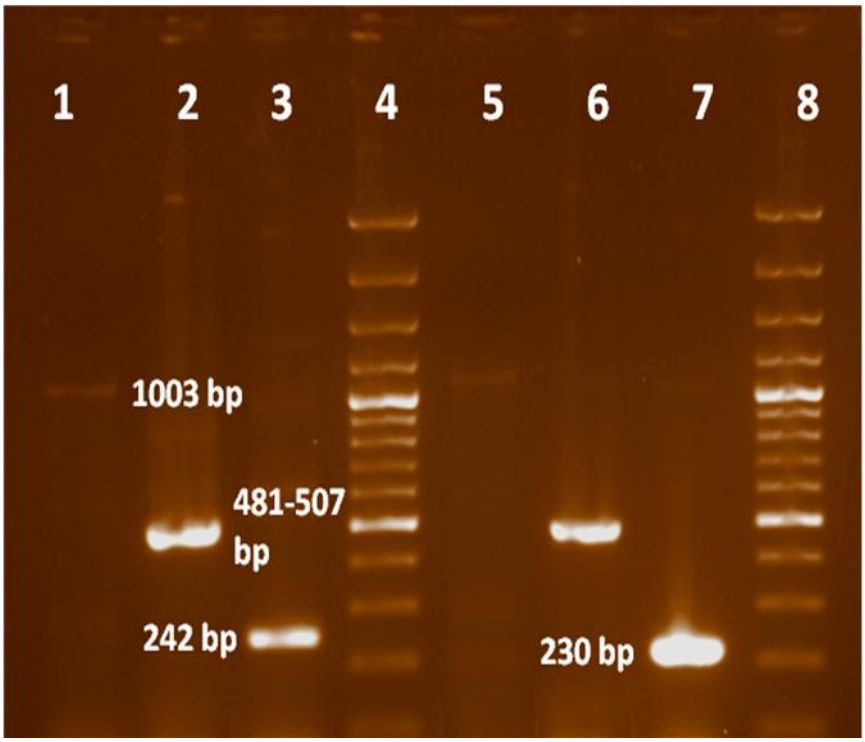


Fig.2 Agarose gel electrophoresis of amplified DNA of *O. tsutsugamushi* by N-PCR



Lane 4 & 8: 100-bp DNA ladder
Lane 1: Positive control showing 1st PCR product of 56KDa surface antigen at 1003-1030 bp
Lane 2: Positive control showing 2nd PCR product of 56KDa surface antigen at 481-507 bp
Lane 3: Positive control showing Kato specific band at 242 bp
Lane 5: Sample showing 1st PCR product at 1003-1030 bp
Lane 6: Sample showing 2nd PCR product at 481-507 bp
Lane 7: Sample showing Karp specific band at 230 bp

Fig.3 (A) Eschar on back region (B) Eschar on chest (C) Eschar on trunk (D) Eschar on inguinal region

Table.1 Nested PCR primer sequences for confirmation of scrub typhus using 56KDa type specific antigen gene (Furuya *et al.*, 1993)

Primer	Sequence (5'-3')	Amplicon size
1st primer set	34-TCAAGCTTATTGCTAGTGCAATGTCTGC 55-AGGGATCCCTGCTGCTGTGCTTGCTGCG	1003-1030 bp
2nd primer set	10-GATCAAGCTlTCCTCAGCCTACTATAATGCC 11-CTAGGGATCCCGACAGATGCACTATTAGGC	481-507 bp

Table.2 Nested PCR primer sequences for strain identification of *O. tsutsugamushi* (Furuya *et al.*, 1993)

Primer	Sequence (5'-3')	Amplicon size
Forward	10-GATCAAGCTlTCCTCAGCCTACTATAATGCC	
Gilliam	G-CTTATATCACTATATATCT	407 bp
Karp	KP-ACAATATCGGATATAACC	230 bp
Kato	KT-GAATAT'ITAATAGCACTGGA	242 bp
Kawasaki	KW-ATGCTGCTATTGATACAGGC	523 bp
Kuroki	KR-CACCGGATITACCATCATAT	407 bp

Table.3 Standardized thermal cycling conditions for 56 Kda and strain specific genes

Initial Denaturation	94^0 c / 5 min	First Cycle
Final Denaturation	94^0 c / 30 sec	35 Cycles
Annealing	55^0 c / 2 min	
Initial Extension	72^0 c / 2 min	
Final Extension	72^0 c / 10 min	Last Cycle
Hold/ Stand by	4^0 c	

Among the three diagnostic methods used in the present study, a good agreement was found between WF test and ICT when compared to N-PCR.

Hence, WF and ICT tests can be used in laboratories where PCR is not available. WF test is inexpensive, easy to perform, and results are available overnight; however, it lacks specificity and sensitivity (Dumler and Siberry, 2007 and Hornick, 2000). ICT is easy to perform and results are available instantly but it is expensive. Gurung *et al.,* (2013*)* and Usha (2014) reported that more scrub typhus cases were positive by ICT tests than WF test.

Since, serological tests may be negative during the early stages of the disease as antibodies are detectable only during the second week of illness (Amano *et al.*, 1992). Hence, frequent follow-up tests are needed. PCR methods when used in conjugation with serological tests can be employed as a specific diagnostic tool for scrub typhus in developing countries (Bakshi *et al.*, 2007) and PCR is considered to be both sensitive and specific method for diagnosis of scrub typhus (Furuya *et al.*, 1993).

This study emphasizes the prevalence of scrub typhus in rural areas where scrub vegetation is more during rainy season. In places where

proper diagnostic facilities are not available, the patients suffering with fever, respiratory symptoms and hepatitis may be suspected for scrub typhus and treated with doxycycline which is the drug of choice for scrub typhus to avoid the associated complications. Also it is one of the misdiagnosed febrile diseases due to non-specific symptoms. Hence, in areas where proper diagnostic facilities are not available, WF can be used as a screening test for preliminary diagnosis of scrub typhus since it is inexpensive and results can be obtained much earlier when compared to N-PCR.

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