

Original Research Article

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

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### ABSTRACT

#### Keywords

ICT, Nested PCR, Scrub typhus and Weil Felix

#### Article Info

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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

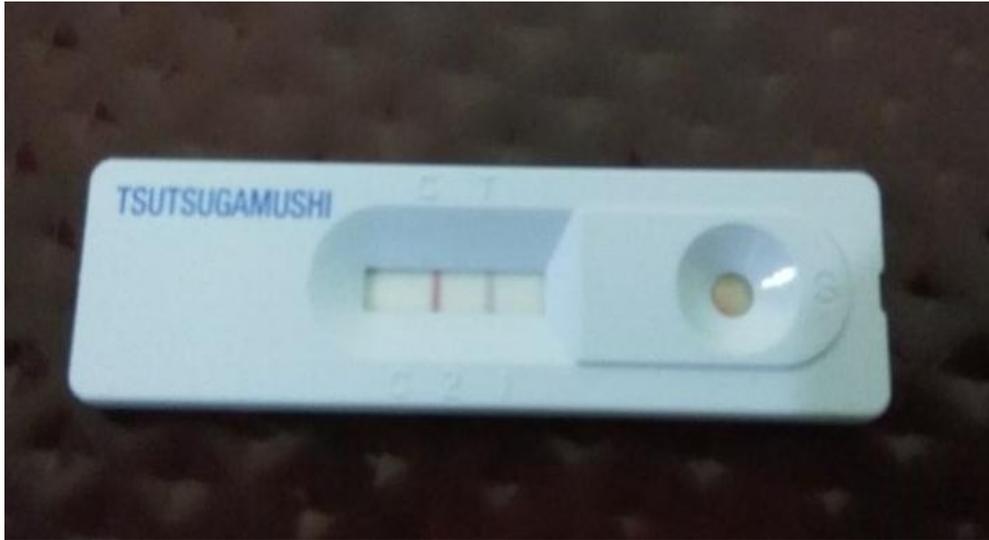
Of the 46 suspected human blood samples, 13 (28.2%) were found serologically positive by both WF and ICT (Fig. 1) methods. The titer more than or equal to 1:160 was taken as positive for WF reaction. 1:160 titer was found in 2 patients, 1:320 in 3, 1:640 in 2, 1:1280 in 4 and 1:2560 in 2 patients.

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

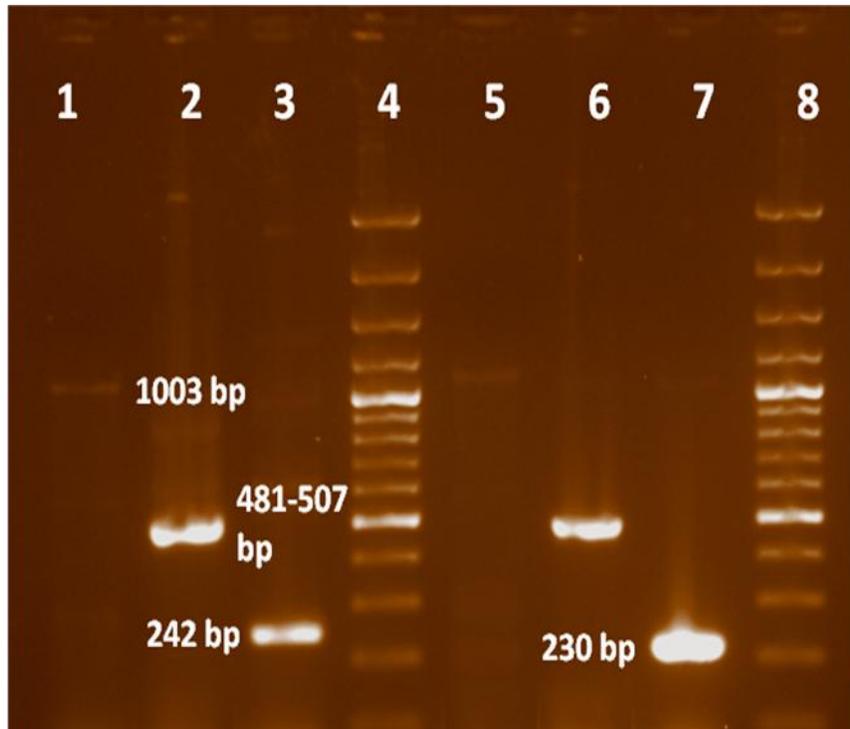
cases were reported to be suffering with fever, headache, myalgia and 46% (6/13) of them were presented with jaundice, 31% (4/13) with dyspnoea, 23% (3/13) with kidney failure, 15% (2/13) with thrombocytopenia and 15% (2/13) with hypotension. Eschar was seen in 4 patients out of 13 confirmed cases (Fig. 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<sub>1</sub> 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 1<sup>st</sup> PCR product of 56KDa surface antigen at 1003-1030 bp

Lane 2: Positive control showing 2<sup>nd</sup> 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 1<sup>st</sup> PCR product at 1003-1030 bp

Lane 6: Sample showing 2<sup>nd</sup> 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



A



B



C



D

**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
<b>1<sup>st</sup> primer set</b>	34-TCAAGCTTATTGCTAGTGCAATGTCTGC 55-AGGGATCCCTGCTGCTGTGCTTGCTGCG	1003-1030 bp
<b>2<sup>nd</sup> primer set</b>	10-GATCAAGCTITCCTCAGCCTACTATAATGCC 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
<b>Forward</b>	10-GATCAAGCTITCCTCAGCCTACTATAATGCC	
<b>Gilliam</b>	G-CTTATATCACTATATATCT	407 bp
<b>Karp</b>	KP-ACAATATCGGATATAACC	230 bp
<b>Kato</b>	KT-GAATAT'ITAATAGCACTGGA	242 bp
<b>Kawasaki</b>	KW-ATGCTGCTATTGATACAGGC	523 bp
<b>Kuroki</b>	KR-CACCGGATITACCATCATAT	407 bp

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

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