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Molecular detection of scrub typhus in Tirupati, Andhra Pradesh, India

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Scrub typhus is an acute rickettsial infectious disease caused by *Orientia tsutsugamushi*, a gram-negative intracellular coccobacillus. It is transmitted to humans and other vertebrates through the bite of the larval trombiculid mites (known as Chiggers) harbouring the etiological agent. The mites normally feed upon single vertebrate hosts, usually rodents. Febrile illness typically begins after the bite of an infected chigger and lasts for 7–10 days[1]. Headache, lymphadenopathy, hepatosplenomegaly, rash, and an eschar usually develop in the infected person. Scrub typhus have been reported from Southeast Asia, Japan, Malaysia, Kampuchea, Thailand, Vietnam, Southeastern Siberia, Sri Lanka, Indonesia, Philippines, Korea, Western Pacific Islands, Pakistan, Astrakhan, India, and northern Australia forming the so called "*tsutsugamushi* triangle" [2-4]. In India, epidemics of scrub typhus have been reported from states of Andhra Pradesh and neighbouring states, as well as from the sub-Himalayan states[5-8]. The aim of the present study was to diagnose the clinically suspected scrub typhus patients serologically by Weil-Felix (WF) test, IgM ELISA and nested-PCR targeting 56-kDa type-specific antigen gene.

From January 2013 to July 2014, 113 blood samples of patients clinically suspected to have scrub typhus, attending Sri Venkateswara Institute of Medical Sciences (SVIMS), Tirupati, India were included in the study. Patients presented with intermittent fever and having five out of the following eight clinical features, namely headache, myalgia, malaise, cough, nausea, abdominal pain, lymphadenopathy, hepatomegaly, splenomegaly, rash and eschar were included in the study[9]. Two ml of blood samples were collected without anticoagulant before the administration of empirical treatment with antibiotics. The Institutional Ethical Clearance was taken from Institute Ethics Committee, SVIMS, Tirupati, India. Serum and blood clot were separated and preserved at –80°C until processing. Demographic details of patients, the clinical course of illness and complications of infection were reviewed from the medical records.

The samples were tested serologically by two techniques. The WF test was performed with each sample by using in-house prepared *Proteus mirabilis* OX-K antigens. Detection of IgM antibodies against *O. tsutsugamushi* was performed by commercial ELISA kit (InBiOS International Inc., USA) as per manufacturer's instructions. The IgM ELISA test was initially standardized using serum samples from healthy blood donors and the OD cut-off of 0.5 was taken 3±SD from the mean. Further, validation was done using known scrub typhus sera (confirmed by PCR) and sera from patients with other diseases like malaria and enteric fever and also healthy controls. A positive and negative control provided in the kit as well as in-house positive control for every run were used. Molecular analysis of the serologically positive samples were carried at Department of Virology, Sri Venkateswara University (SVU), Tirupati, India. DNA was extracted from blood clot of each sample by using the method as described by Furuya *et al*[10]. The 56-kDa type-specific antigen (TSA) gene was amplified by nested-PCR (N-PCR) using the following primers:

Primer set 1:

$_P$35-5' TCA AGC TTA TTG CTA GTG CAA TGT CTG C 3'

$_P$55-5' AGG GAT CCC TGC TGC TGT GCT TGC TGC G 3'

Primer set 2:

$_P$10-5' GAT CAA GCT TCC TCA GCC TAC TAT AAT GCC 3'

$_P$11-5' CTA GGG ATC CCG ACA GAT GCA CTA TTA GGC 3'

Primer set 1 amplified a 1003 bp fragment and primer set 2 amplified 483 bp fragment. PCR products were electrophoresed in 1% agarose gel containing ethidium bromide (0.5 µg/ml) and visualized using a gel documentation system (BioRad, USA). The expected 483-bp products were purified by using QIAquick PCR purification kit (Qiagen, USA) according to the manufacturer's instructions. The purified product was sequenced by Sanger's dideoxy method on ABI 3730 × l automated sequencer. Samples were selected for sequencing, based

on demographic criteria. Nucleotide sequences of *O. tsutsugamushi* detected in this study were deposited in GenBank under accession numbers KJ094995, KJ740606, and KJ740607. Database homology search with each obtained sequence was done using GenBank (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*). Complete or partial sequences encoding 56-kDa TSA of *O. tsutsugamushi* reference strains were retrieved from the GenBank. Multiple sequence alignment and analysis of the genetic relationships between study isolated strains and reference strains were performed using BioNumerics version 4.6 Clustal X algorithm. Phylogenetic relationships among the study sequences and reference sequences were constructed with software programme MEGA version 4 by neighbour-joining method. Reliability of the phylogenetic analysis was evaluated using the bootstrap test on 1000 replicates.

Of the 113 clinically suspected scrub typhus patients, majority of the patients were males 66 (58.40%) compared to females 47 (41.59%). The mean age (± standard deviation) of the clinically suspected patients was 44.47 ± 19.26 (median, 46 yr). Majority of the patients (73, 64.6%) were from rural areas. Most of the patients belonged to Chittoor district (79, 69.91%), followed by Kadapa (17, 15.04%), Nellore (14, 12.38%) and Anantapur (3, 2.65%) districts of Andhra Pradesh. Among the studied population most of the patients were agricultural workers (50, 44.24%) followed by their household members (29, 25.66%), labourers (22, 19.46%) and others (12, 10.61%). In N-PCR positive patients, headache (19, 47.5%) was the common feature followed by cough (18, 45%), hepatomegaly (11, 27.5%), lymphadenopathy (10, 25%), myalgia (10, 25%), abdominal pain (8, 20%), and splenomegaly (8, 20%). Eschar (5, 12.5%) was significantly ($p = 0.002$) more common in those with scrub typhus.

Among the 113 clinically suspected scrub typhus patients, 44 (38.93%) patients were found to be positive by WF test (Titer ≥1 in 80). A titer of 1 : 80 was seen in six patients, 16 patients showed a titer of 1 : 160 and a titer of 1 : 320 was seen in 19 patients against *P. mirabilis* OX-K. Titer of 1 : 1280 was noted only in three patients against *P. mirabilis* OX-K.

Similarly, by ELISA, IgM antibodies to the 56-kDa antigen were detected in 40 (35.39%) out of 113 patients. All the samples were further subjected to N-PCR analysis. Of the 113 samples tested, 40 (35.39%) samples showed 483 bp DNA fragment corresponding to 56-kDa gene on the agarose gel. It was observed that all the positive samples by IgM ELISA were also confirmed by N-PCR; while four samples positive to WF test, were not amplified by N-PCR.

Weil-Felix test showed more positives when compared with ELISA and N-PCR tests. It was also found that there was good agreement between WF test and ELISA when compared with N-PCR. So, WF and ELISA tests can be used in laboratories where PCR is not available. The scrub typhus ELISA is a flexible alternative to the IFA technique[11] and have specificities and sensitivities of >90% for detecting specific-antibodies[12]. The ELISA format is very convenient for large-scale testing in laboratory and takes about 50 min to perform[13]. Since, serological tests have low sensitivities in the early stage of scrub typhus due to insufficient production of antibodies, frequent follow-up tests are needed[14]. PCR methods when used independently or in conjugation with WF test can be employed as a specific diagnostic tool for diagnosis of scrub typhus in developing countries, aiding in the surveillance and effective treatment of this emerging infectious disease[15].

All the WF positive samples which were tested positive by N-PCR had titer of 1 : 160 or above whereas those which tested negative had a titer of 1 : 80. The strains obtained in our study, namely SVU/SVIMS CTR, SVU/SVIMS ANTPR, and SVU/SVIMS 28 formed a cluster close to Japanese Gilliam (JG) type comprising of Hualien-7, Taitung-2 and UT329 (Fig. 1).

During the study period it was found that most of the positive scrub typhus cases occurred during the months of October to December. Clinically suspected cases were tested by WF and IgM ELISA tests. Serologically positive samples were further confirmed by N-PCR assay. Despite of growing knowledge on epidemiology and phylogeny of *Orientia* strains in endemic regions, there is a true paucity of information from India. For genotyping we have targeted the 56-kDa protein gene as it is a major outer membrane protein containing both group-specific and type-specific epitopes, which are useful for diagnosis of scrub typhus[16]. The genotyping method also has the advantage of detecting unknown serotypes that are not identified by assays based on reactions with monoclonal antibodies against known strains[17].

In the present investigation, we found that our study strains were similar to JG type that refers to "Gilliam type" in Japan on the basis of serological cross-reactions[18]. But by the analysis of sequences by sequence similarity, JG was found closely related to Kawasaki, but not to Gilliam and forms significant group within *Orientia* species. Kawasaki type sequences have been found in Japan and China, whereas JG and JG-v sequences have been reported from Japan, China, and Southeast Asia[19]. A study from Himachal Pradesh, India reported that their study strain sequences (ISS-1 and 2) closely matched the Japanese

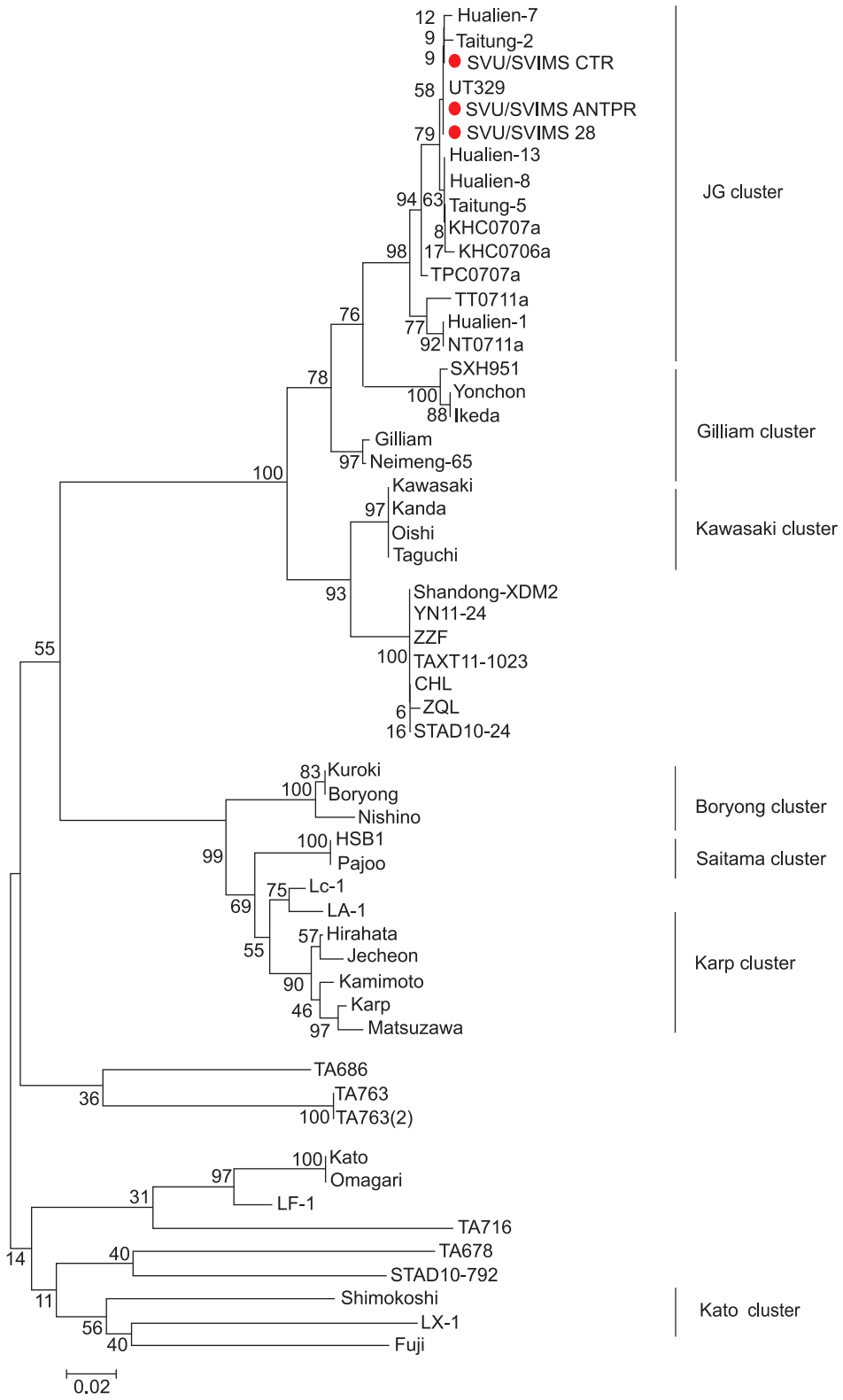

Fig. 1: Phylogenetic tree-based on nucleotide sequences of *O. tsutsugamushi* type-specific antigens.

Gilliam cluster[15]. In another study from India, among the two new genotypes that were causing scrub typhus in Himalayan regions, the phylogenetic position of one was between Karp and JP-1 while the other was between Saitama and JG type[20]. Karp and Kato like strains have been reported in Vellore, Tamil Nadu by Vargheese *et*

al[21]. In conclusion, it can be said that there exists genotypic variation among the strains prevalent in India which needs further evaluation.

REFERENCES

1. Rosenberg R. Drug resistant scrub typhus: Paradigm and paradox. *Parasitol Today* 1997; *13*(4): 131–2.
2. Shirai A, Robinson DM, Brown GW, Gan E, Huxsoll DL. Antigenic analysis by direct immunofluorescence of 114 isolates of *Rickettsia tsutsugamushi* recovered from febrile patients in rural Malaysia. *Jpn J Med Sci Biol* 1979; *32*(6): 337–44.
3. Berman SJ, Kundin WD. Scrub typhus in South Vietnam. A study of 87 cases. *Ann Intern Med* 1973; *79*(1): 26–30.
4. Yamashita T, Kasuya S, Noda N, Nagano I, Kang JS. Transmission of *Rickettsia tsutsugamushi* strains among humans, wild rodents, and trombiculid mites in an area of Japan in an area in which *tsutsugamushi* disease is newly endemic. *J Clin Microbiol* 1994; *32*(11): 2780–5.
5. Mathai E, Lloyd G, Cherian T, Abraham OC, Cherian AM. Serological evidence for the continued presence of human rickettsioses in southern India. *Ann Trop Med Parasitol* 2001; *95:* 395–8.
6. Sharma A, Mahajan S, Gupta ML, Kanga A, Sharma V. Investigation of an outbreak of scrub typhus in the Himalayan region of India. *Jpn J Infect Dis* 2005; *58*(4): 208–10.
7. Mahajan SK, Rolain JM, Sankhyan N, Kaushal RK, Raoult D. Pediatric scrub typhus in Indian Himalayas. *Indian J Pediatr* 2008; *75*(9): 947–9.
8. Vivekanandan M, Mani A, Priya YS, Singh AP, Jayakumar S, Purty S. Outbreak of scrub typhus in Pondicherry. *J Assoc Physicians India* 2010; *58:* 24–8.
9. Premaratna R, Loftis AD, Chandrasena TG, Dasch GA, de Silva HJ. Rickettsial infections and their clinical presentations in the Western Province of Sri Lanka: A hospital-based study. *Int J Infect Dis* 2008; *12*(2): 198–202.
10. Furuya Y, Yoshida Y, Katayama T, Yamamoto S, Kawamura A Jr. Serotype-specific amplification of *Rickettsia tsutsugamushi* DNA by nested polymerase chain reaction. *J Clin Microbiol* 1993; *31:* 1637–40.
11. Dasch GA, Halle S, Bourgeois AL, Sensitive microplate enzyme linked immunosorbent assay for detection of antibodies against the scrub typhus rickettsia, *Rickettsia tsutsugamushi*. *J Clin Microbiol* 1979; *9*(1): 38–48.
12. Varghese GM, Abraham, MOC, Mathai D, Thomas K, Aaron R, Kavitha ML, *et al*. Scrub typhus among hospitalized patients with febrile illness in south India: Magnitude and clinical predictors. *J Infect* 2006; *52*(1): 56–60.
13. Ching WM, Rowland D, Zhang Z, Bourgeois AL, Kelly D, Dasch GA, *et al*. Early diagnosis of scrub typhus with a rapid flow assay using recombinant major outer membrane protein antigen (r56) of *Orientia tsutsugamushi*. *Clin Diagn Lab Immunol* 2001; *8*(2): 409–14.
14. Bozeman FM, Elisberg BL. Serological diagnosis of scrub typhus by indirect immunofluorescence. *Proc Soc Exp Biol Med* 1963; *112:* 568–73.
15. Bakshi D, Singhal P, Mahajan SK, Subramaniam P, Tuteja U, Batra HV. Development of a real-time PCR assay for the diagnosis of scrub typhus cases in India and evidence of the prevalence of new genotype of *O. tsutsugamushi*. *Acta Trop* 2007; *104*(1): 63–71.
16. Ohashi N, Nashimoto H, Ikeda H, Tamura A. Cloning and sequencing of the gene (tsg56) encoding a type-specific antigen from *Rickettsia tsutsugamushi*. *Gene* 1990; *91:* 119–22.
17. Park SW, Lee CK, Kwak YG, Moon C, Kim BN, Kim ES, *et al*. Antigenic drift of *Orientia tsutsugamushi* in South Korea as identified by the sequence analysis of a 56-kDa protein-encoding gene. *Am J Trop Med Hyg* 2010; *83*(4): 930–5.
18. Tamura A, Yamamoto N, Koyama S, Makisaka Y, Takahashi M, Urabe K, *et al*. Epidemiological survey of *Orientia tsutsugamushi* distribution in field rodents in Saitama Prefecture, Japan, and discovery of a new type. *Microbiol Immunol* 2001; *45*(6): 439–46.
19. Kelly DJ, Fuerst PA, Ching WM, Richards AL. Scrub typhus: The geographic distribution of phenotypic and genotypic variants of *Orientia tsutsugamushi*. *Clin Infect Dis* 2009; *48:* 203–30.
20. Mahajan SK, Rolain JM, Kashyap R, Bakshi D, Sharma V, Prasher BS, *et al*. Scrub typhus in Himalayas. *Emerg Infect Dis* 2006; *12*(10): 1590–2.
21. Varghese GM, Janardhanan J, Trowbridge P, Peter JV, Prakash JA, Sathyendra S, *et al*. Scrub typhus in south India: Clinical and laboratory manifestations, genetic variability, and outcome. *Int J Infect Dis* 2013; *17*(11): e981–7.

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