International Journal of Microbiology Research and Reviews ISSN 2329-9800 Vol. 4 (6), pp. 158-160, June, 2015. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research paper

Molecular diagnosis of scrub typhus: A preliminary report from Pondicherry

KS Roopa[1], K Karthika[2] and BN Harish*

[1] PhD scholar, Department of Microbiology, Jawaharlal Institute of Post Graduate Medical Education and Research,
[2] Senior Research fellow, ICMR, Department of Microbiology, Jawaharlal Institute of Post Graduate Medical Education and Research.
*Senior Professor and Head, Department of Microbiology, Jawaharlal Institute of Post Graduate Medical Education and Research, Gorimedu, Puducherry -605006, India. Email: drbnharish@gmail.com

Accepted 20 May, 2015

Scrub typhus is a febrile, zoonotic disease caused by the organism *Orientia tsutsugamushi*. Early and precise diagnosis is crucial to reduce the risk of complications of the disease and prevent mortality. Although serology has remained a mainstay for diagnosis it does not help initial treatment. Nested PCR offers an early and viable alternative. The objective of our study is to standardise nested PCR for 56 kDa gene of *Orientia tsutsugamushi* in the diagnosis of scrub typhus. Nested PCR was performed on 406 EDTA blood samples with clinically suspected scrub typhus to detect 56kDa gene of *Orientia tsutsugamushi.* Weil-Felix test was performed on serum samples for the same patients. Among 406 patients, 51(12.56%) showed positive by Weil-Felix test against OX-K antigen and 91(22.41%) showed positive by nested PCR. Among 91 cases which were positive by nested PCR, 68 (74.73%) showed negative by Weil-Felix test. Out of 51 cases positive by Weil-Felix test, 28 (54.90%) cases showed negative by nested PCR. Nested PCR targeting the *Orientia tsutsugamushi* specific for 56kDa gene acts as an improved diagnostic tool than Weil-Felix test which still remains a mainstay in resource poor settings for diagnosis of scrub typhus.

Keywords: Scrub typhus, 56kDa gene, nested polymerase chain reaction, Weil-Felix test, pondicherry.

INTRODUCTION

Scrub typhus is re-emerging rickettsial infection in recent times. The organism is a gram negative obligate intracellular, nonflagellate, pleomorphic coccobacilli belonging to family Rickettsiaceae, genus *Rickettsia*. This is a zoonotic disease that is transmitted only by the larval stage of mite which is called as a chigger stage. Off late the disease has been urbanised and prevalence has been broadened further, to not only be seen in scrub areas (areas after clearance of primary forest) but also in abandoned plantations, rice fields, river banks, gardens and forests.(Mahajan, 2005; Sharma et al., 2010). Thus, to term it as scrub typhus in the prevailing scenario may be inappropriate.

The disease is a major public health threat in the triangle which extends from Japan and Russia in the North to Northern Australia in the South and the Arabian Peninsula in the West.(Mathai et al., 2001) Many states in India have documented scrub typhus which includes Jammu and Kashmir, Himachal Pradesh, Uttaranchal, Rajasthan, Sikkim, Goa, Assam, West Bengal, Maharashtra, Kerala, Tamil Nadu and Pondicherry (Ahmad et al., 2010; Gurung et al., 2013; Kamarasu et al., 2007; Mittal et al., 2012; Narvencar et al., 2012; Prabagaravarthanan et al., 2008; Saifudheen et al., 2012; Sharma et al., 2005; Usha et al., 2014). The disease accounts for 23% of all febrile episodes in areas of Asian pacific region where scrub typhus is endemic.(Huber et al., 2012) The predominant signs and symptoms include fever, headache, myalgia, and abdominal discomfort. The clinical manifestations are non-specific to make clinical diagnosis. Serology remains the mainstay for diagnosis of scrub typhus. Weil-Felix test is the most common serology test uses the heterophile antigen, the *Proteus vulgaris* which cross reacts with OX19 and OX 2 and the *Proteus mirabilis* that cross reacts with OX K antigen. Although Weil-Felix test is easy to perform, cheap and quick test, it lacks sensitivity (Prakash et al., 2006). The mIFA which is considered as a gold standard for diagnosis of scrub typhus, is expensive and also requires fluorescence microscope and expertise for interpretation

of the result. These antibody based tests may not help in acute cases whereas molecular method like nested PCR offers a viable alternative and therefore the study was undertaken. In our study an attempt has been made to evaluate nested PCR for the diagnosis of scrub typhus in patients with clinical suspision of scrub typhus.

MATERIALS AND METHODS

The study was carried out between January 2012 to December 2014, at tertiary care hospital in Puducherry, South India. The patients attending hospital (both inpatients and outpatients) who were clinically diagnosed to have typhus fever were included in the study. The study includes patients of all age group .The patients with a history of fever ,with or without presence of eschar, maculopapular skin rash and patients with ≥2 symptoms such as headache, myalgia, nausea, abdominal discomfort and malaise were included in the study. Patients who were positive for other febrile agglutination tests like WIDAL, Paul Bunnel etc. and positive for other common causes of fever like filariasis, malaria, dengue, leptospirosis etc. were excluded from the study.

Specimen collection and processing:

The blood samples were collected from all febrile patients who visited JIPMER hospital during the study period and were clinically suspected as typhus fever. Five millilitre of venous blood sample was collected from each patient. The blood was collected in two containers one with EDTA for nested PCR and the other in plain tube for Weil-Felix test. The study was conducted only after obtaining written informed consent from the patients. The approval for the study was taken from JIPMER scientific advisory committee and JIPMER Institute Ethics committee.

Weil-Felix tube agglutination test

Weil-Felix tube agglutination test was performed for all samples using the commercial kit (Plasmatech, UK) as per the manufacturer's instructions. Agglutination titres of ≥160 to OX K antigen were taken as positive for scrub typhus.

Nested PCR:

Genomic DNA was isolated from the EDTA treated blood samples. DNA extraction was carried out using QIAamp DNA mini kit (Qiagen. Hilden, Germany) as per the manufacturer's instructions. A final volume of 30µl of DNA was eluted and was stored at -70ºC till nested PCR was carried out.

Nested polymerase chain reaction involves two sets of primers, used in two successive runs of polymerase chain reaction, the second set intended to amplify a secondary target with the first run product.

The primers were taken from Furuya *et al.*, 1993. (Furuya et al., 1993). The primers binds and amplifies specifically a portion of 56kDa gene (major immunogenic antigen) of *Orientia tsutsugamushi*, the causative agent of scrub typhus. The nested PCR were carried out using commercially available master mix (Taqman 2X master mix: Red, Ampliqon). The first step of nested PCR (50µl of PCR amplification mixture) contained 15µl of ampliqon master mix, 1µl each of 10 pmole primers namely, p55 and p34 (Eurofins, Bangalore, India), 3µl of extracted DNA and 30 µl autoclaved milliQ water. Three µl product of the first step of nested PCR was used as template DNA for the second step of nested PCR and 10pmole of primers namely; p10 and p11 were used instead. The primers used are given below:

Outer set of primers:
p34 (5'-TCAAGCTTATTGCTAGTGCAATGTCTGC-3')
p55 (5'-AGGGATCCCTGCTGCTGTGCTTGCTGCG-3')
Inner set of primers:
p10 (5'-GATCAAGCTTCCTCAGCCTACTATAATGCC-3')
p11 (5'-CTAGGGATCCCGACAGATGCACTATTAGGC-3')

The PCR conditions for first and second step of nested PCR remained the same and were as follows:Initial denaturation at 94ºC for 30 sec, followed by denaturation, annealing and extension at 94ºC for 30 sec, 57ºC for 2 minutes and 70ºC for 2 minutes, respectively, for a total of 30 cycles followed by final extension at 70ºC for 10 minutes. The PCR reaction was carried out in a thermocycler (Eppendorf). The PCR product was electrophoresed in 1.5% agarose gel containing ethidium bromide (0.5mg/ml) and 483bp PCR product was visualised under UV transilluminator.

Nested PCR amplicon sequencing carried out to confirm the identity of 56kDa gene of *Orientia tsutsugamushi:* Two nested PCR amplicons were sequenced to confirm the identity of the 56kDa gene of *Orientia tsutsugamushi*. The sequencing was carried out commercially (Eurofins, Sequencer - Applied Bio systems 3730, Bangalore, India).

RESULTS

A total of 451 patients with acute febrile illness were screened with a battery of diagnostic tests such as WIDAL tests, Ig M ELISA for leptospirosis, ICT for dengue and Para F for malaria. Out of which 45 samples reacted positively for above mentioned tests and were excluded from the study. Out of 406 patients, 248 (61%) were males and 158(39%) were females. Among 406 patients, 51(12.56%) showed positive by Weil-Felix test against OX K antigen and 91(22.41%) showed positive by nested PCR. Among 91 cases which were positive by nested PCR, 68 (74.73%) showed negative by Weil-Felix test. Out of 51 cases positive by Weil-Felix test, 28

Table 1. Results of nested PCR and Weil-Felix tests in scrub typhus diagnosis (n=406).

	Weil-Felix test (reactive to OX K antigen)	Weil-Felix test (non-reactive to OX K antigen)
Nested PCR positive	23	68
Nested PCR negative	28	287
Total no. of samples	406	

(54.90%) cases showed negative by nested PCR as showed in table 1.

Two nested PCR amplicons were sequenced and found to have a similarity of 99% with *Orientia tsutsugamushi* clone ISS-11 56 kDa type specific antigen gene, partial cds (Sequence ID: gb|DQ286233.1|) with a length of 460 bp.

DISCUSSION

Early and accurate diagnosis of scrub typhus is crucial to guide appropriate antimicrobial therapy and patient management to reduce the chance of life-threatening complications. The disease is misdiagnosed due to lack of awareness among the clinicians, low index of suspicion, protean clinical manifestation and non-availability of accurate diagnostic test. The pathognomonic eschar and rash is seen only in less than 50% of the cases making the clinical diagnosis difficult. (Mathai et al., 2003). Moreover, skin manifestations may go unnoticed in the dark skinned people. Therefore laboratory tests are required to make a diagnosis. Though serology has remained a mainstay for diagnosis of scrub typhus, it is bound with limitations. Weil-Felix test being the most common of all serological tests lacks sensitivity (Prakash et al., 2006). The immunofluorescence antibody test which is considered as a gold standard requires an expensive fluorescence microscope and also the commercially available kit has antigens only for strains Gilliam, Karp, Kato and Boryong that cannot detect other strains present in different geographical location and hence the sensitivity of the test is reduced. The interpretation of IFA test is subjective and requires technical expertise for performance of the test. (Prakash et al., 2006). The culturing of the organism *O.tsutsugamushi* is notoriously dangerous requiring bio-safety level III containment facility which is unavailable in most laboratories and also requires experienced personnel.

The prime advantage of using molecular based technique is that, the detection of the disease is possible even before antibody is detected by serological method. The method is excellent diagnostic tool in such a circumstance where culture of the organism is difficult and serological test diagnose lately (end of first week of infection). Nested PCR is popularly used molecular technique which also helps in early diagnosis of scrub typhus detecting the *O. tsutsugamushi* DNA within two days of onset of fever. Thus helps the clinicians to start appropriate antibiotics in the early stage of infection, when the antibiotic therapy is most effective.

In previous study it was reported that the 56kDa protein gene seems to be is more useful in differentiating 16s rRNA gene from 56 kDa gene. The 16s rRNA genes of Gilliam, Karp , Kato, and Kuroki strains are ≥98.4% homologous and hence it is useful for differentiating between other genera / species, but not between *O. tsutsugamushi* strains.(Lee et al., 2011). A study carried out by Kim *et al*.(2011) using 41 scrub typhus samples have found the positivity rate for 47kDa gene using conventional PCR, nested PCR and quantitative real time PCR to be 7%, 85% and 83% respectively , while for 56kDa antigen, nested PCR was positive in 87.8% cases. The study also reported that nested PCR is much more sensitive as compared to conventional PCR. (Kim et al., 2011). In 2014, Janardhanan *et al.,* reported that positivity rate for conventional PCR and nested PCR were 95.8% and 75% respectively among 24 patients for 56KDa gene detection using eschar samples. (Janardhanan et al., 2014)

Mathai and his co-workers reported that 6/23(23.1%) cases were negative by Weil-Felix test when compared with micro Immunofluorescence Antibody test (mIFA) which is gold standard for scrub typhus diagnosis signifying that Weil-Felix test is grossly insensitive to anti-tsutsugamushi antibodies and hence it is not useful for screening cases.(Mathai et al., 2003). In our study, nested PCR positivity was 22.41% whereas Weil-Felix test against OX K antigen showed positivity of 12.56%. Hence by performing nested PCR which is an improved diagnostic method will allow greater appreciation of prevalence of the disease. The challenge to the clinicians with regard to the diagnostic predicament posed by these infections in the early stage of the clinical course when antibiotic therapy is most effective will be addressed.

Attempts are being made to standardise Loop Mediated Isothermal Amplification assay (LAMP) in diagnosis of scrub typhus in our laboratory. LAMP assays makes diagnosis simple because of its swiftness, sensitivity and specificity. It is simpler than conventional PCR not requiring thermocycler and post amplification modifications. The assay is carried out at constant temperature unlike the conventional PCR.

Limitation: The main limitation of our study is that correlation between the laboratory finding and clinical outcome is not included.

CONCLUSION

Nested PCR targeting the *O. tsutsugamushi* specific for 56kDa acts as a better diagnostic tool than Weil-Felix test which lacks sensitivity.

Source of support:

1. Jawaharlal Institute of Post Graduate Medical Education and Research, Gorimedu, Puducherry, India,
2. Indian Council of Medical Research (ICMR),New Delhi, India

Conflict of interest: None

Acknowledgement for financial support and specific contribution only: The authors would like to acknowledge the funding agency Indian Council of Medical Research (ICMR),New Delhi, India for their support.

REFERENCES

Ahmad S, Srivastava S, Verma SK, Puri P, Shirazi N (2010). Scrub typhus in Uttarakhand, India: a common rickettsial disease in an uncommon geographical region. Trop. Doct. 40, 188–190.

Furuya Y, Yoshida Y, Katayama T, Yamamoto S, Kawamura A (1993). Serotype-specific amplification of Rickettsia tsutsugamushi DNA by nested polymerase chain reaction. J. Clin. Microbiol. 31, 1637–1640.

Gurung S, Pradhan J, Bhutia PY (2013). Outbreak of scrub typhus in the North East Himalayan region-Sikkim: an emerging threat. Indian J. Med. Microbiol. 31, 72–74.

Huber E, Ji D, Howell L, Zhang Z, Chen H, Ching W, Chao C (2012). Loop-Mediated Isothermal Amplification Assay Targeting the 47-Kda Gene of Orientia tsutsugamushi: A Rapid and Sensitive Alternative to Real-Time PCR. J. Med. Microbiol. Diagn. 01.

Janardhanan J, Prakash JAJ, Abraham OC, Varghese GM (2014). Comparison of a conventional and nested PCR for diagnostic confirmation and genotyping of Orientia tsutsugamushi. Diagn. Microbiol. Infect. Dis. 79, 7–9.

Kamarasu K, Malathi M, Rajagopal V, Subramani K, Jagadeeshramasamy D, Mathai E (2007). Serological evidence for wide distribution of spotted fevers & typhus fever in Tamil Nadu. Indian J. Med. Res. 126, 128–130.

Kim DM, Park G, Kim HS, Lee JY, Neupane GP, Graves S, Stenos J (2011). Comparison of conventional, nested, and real-time quantitative PCR for diagnosis of scrub typhus. J. Clin. Microbiol. 49, 607–612.

Lee YM, Kim DM, Lee SH, Jang MS, Neupane GP (2011). Phylogenetic analysis of the 56 kDa protein genes of Orientia tsutsugamushi in Southwest Area of Korea. Am. J. Trop. Med. Hyg. 84, 250–254.

Mahajan S (2005). Scrub Typhus. J. Assoc. Physicians India 53, 954–958.

Mathai E, Lloyd G, Cherian T, Abraham OC, Cherian AM (2001). Serological evidence for the continued presence of human rickettsioses in southern India. Ann. Trop. Med. Parasitol. 95, 395–398.

Mathai E, Rolain JM, Verghese GM, Abraham OC, Mathai D, Mathai M, Raoult D (2003). Outbreak of scrub typhus in southern India during the cooler months. Ann. N. Y. Acad. Sci. 990, 359–364.

Mittal V, Gupta N, Bhattacharya D, Kumar K, Ichhpujani RL, Singh S, Chhabra M, Rana UVS (2012). Serological evidence of rickettsial infections in Delhi. Indian J. Med. Res. 135, 538–541.

Narvencar KPS, Rodrigues S, Nevrekar RP, Dias L, Dias A, Vaz M, Gomes E (2012). Scrub typhus in patients reporting with acute febrile illness at a tertiary health care institution in Goa. Indian J. Med. Res. 136, 1020–1024.

Prabagaravarthanan R, Harish BN, Parija SC (2008). Typhus fever in Pondicherry. J. Commun. Dis. 40, 159–160.

Prakash JAJ, Abraham OC, Mathai E (2006). Evaluation of tests for serological diagnosis of scrub typhus. Trop. Doct. 36, 212–213.

Saifudheen K, Kumar KGS, Jose J, Veena V, Gafoor VA (2012). First case of scrub typhus with meningoencephalitis from Kerala: An emerging infectious threat. Ann. Indian Acad. Neurol. 15, 141–144.

Sharma A, Mahajan S, Gupta ML, Kanga A, Sharma V (2005). Investigation of an outbreak of scrub typhus in the himalayan region of India. Jpn. J. Infect. Dis. 58, 208–210.

Sharma P, Kakkar R, Shilpa NK, Yadav VK, Sharma R (2010). Geographical Distribution, Effect of Season & Life Cycle of Scrub Typhus. JK Science 12, 63–64.

Usha K, Kumar E, Kalawat U, Kumar BS, Chaudhury A, Gopal DS (2014). SeroprevalenceOf Scrub Typhus Among Febrile Patients: A Preliminary Study. Asian J. Pharm. Clin.Res. 7.