Indian Journal of Medical Microbiology, (2011) 29(1): 47-50

Original Article

Nested polymerase chain reaction on blood clots for gene encoding 56 kDa antigen and serology for the diagnosis of scrub typhus

***JAJ Prakash, ML Kavitha, E Mathai**

Abstract

Purpose: Scrub typhus is a zoonotic illness endemic in the Asia-Pacific region. Early diagnosis and appropriate management contribute significantly to preventing adverse outcomes including mortality. Serology is widely used for diagnosing scrub typhus. Recent reports suggest that polymerase chain reaction (PCR) could be a rapid and reliable alternative. This study assessed the utility of these tests for scrub typhus diagnosis. **Materials and Methods**: Nested PCR to detect the 56 kDa antigen gene of *O. tsutsugamushi* was performed on blood clots from 87 individuals with clinically suspected scrub typhus. Weil-Felix test and scrub typhus IgM ELISA were performed on serum samples from the same patients. As a gold standard reference test was not available, latent class analysis (LCA) was used to assess the performance of the three tests. **Results**: The LCA analysis showed the sensitivity of Weil-Felix test, IgM ELISA and PCR to be 59%, 100% and 58% respectively. The specificity of ELISA was only 73%, whereas those of the Weil-Felix test and PCR were 94% and 100% respectively. **Conclusion**: Nested PCR using blood clots while specific, lacked sensitivity as compared to IgM ELISA. In resource-poor settings Weil-Felix test still remains valuable despite its moderate sensitivity.

Key words: *56 kDa gene, blood clot, nested polymerase chain reaction, scrub typhus, serology*

Introduction

Scrub typhus, a zoonosis endemic in the Asia-Pacific region, is caused by *O. tsutsugamushi*, and transmitted by larvae of trombiculid mites. It has re-merged as a major cause of fever of unknown origin in peninsular India in recent years. Mortality rates approaching 30% are reported.[1]

Scrub typhus is usually diagnosed based on clinical presentation and serology. Isolation of the causative organisms is done only in specialised reference laboratories. Though the indirect immunofluorescence assay is the reference standard,[2] Weil-Felix test has remained the mainstay of the serodiagnosis of scrub typhus in India as the antigen is easily available and it is cheaper and easier to perform.[3] A commercially available IgM ELISA, which uses a standardized, recombinant antigen has been found to be suitable and recommended for use in a diagnostic laboratory.[4] An evaluation of this ELISA (Panbio Ltd, Brisbane, Australia) demonstrated a sensitivity and specificity of 86.5% and 97.5% respectively.[5] However, false positive results are not uncommon.

Nested and non-nested polymerase chain reaction (PCR) formats using various gene targets have been described for the diagnosis of scrub typhus. The primers described by Furuya and co-workers to detect the gene encoding the immunodominant 56 kDa gene[6] have been used in nested PCR on blood clots,[7] buffy coat,[8] EDTA blood[9] and eschars.[10,11] Sonthayanon *et al.*, designed and evaluated an assay which detects the 16S rRNA gene of *O. tsutsugamushi*.[9] A non-nested PCR was used by Mahajan *et al.*, to amplify *O. tsutsugamushi* DNA leading to the subsequent identification of two new genotypes in Himachal Pradesh.[12] In addition, quantitative real-time PCR assays to detect the 47 kDa gene[13] and 16 S rRNA,[14] 56 kDa gene[15] and the groEL gene[16] have also been assessed for their efficiency in the detection of *O. tsutsugamushi* DNA and measurement of bacterial load.

The objective of this study was to evaluate the nested PCR using primers described by Furuya for the laboratory diagnosis of scrub typhus in our population. These primers were chosen as they encode for the gene encoding the immunodominant 56 kDa protein, which is also the antigen used for detection of IgM antibodies in the ELISA routinely used in our laboratory for serological diagnosis of scrub typhus.

*Corresponding author (email:<prakjaj@yahoo.com>)
Departments of Microbiology (JAJP, EM) and Haematology (MLK), Christian Medical College, Vellore - 632 004, India.
Received: 27-11-2010
Accepted: 18-01-2011

Access this article online

Quick Response Code:

Website: www.ijmm.org

DOI: 10.4103/0255-0857.76524

Materials and Methods

Consent for this study was obtained from the institutional review board (IRB) of our hospital.

Samples

Venous blood (5 ml) samples from 87 patients, who presented with an acute febrile illness to the hospital of a tertiary care centre in India between January 2004 and March 2006, were used for the study. Clinically, scrub typhus was suspected in all these patients.

Serology

Serum was separated by centrifugation at 2500 g for 10 min and the clots stored at -70°C pending DNA extraction. The serum samples were tested by Weil-Felix (WF) test using in-house reagents as previously reported[5] and by IgM ELISA (Panbio Ltd, Brisbane, Australia). A titre of ≥ 80 for OX K antigen and ≥ 16 units in IgM ELISA were considered to be positive results.

Nested polymerase chain reaction

DNA was extracted from 200 µl of homogenized clotted blood using the QIAmp blood mini kit (Qiagen GmBh, Germany) as per manufacturer's instructions. The purified DNA was aliquoted and stored at -70°C. DNA amplification was performed using a nested format. The primers used in this study were described in 1993 by Furuya *et al.,*[6] and reported by Saisongkorh and co-workers in 2004.[7] A 483 bp segment of the gene encoding the 56 kDa antigen of *O. tsutsugamushi* was amplified by nested PCR and the primer sequences (Sigma-Aldrich, Bangalore, India) used are as given below:

Outer primer set

P55: 5'-TCA AGC TTA TTG CTA GTG CAA TGT CTGC- 3'
P34: 5' –AGG GAT CCC TGC TGC TGT GCT TGC TGCG-3'
Inner primer set
P10: 5'-GAT CAA GCT TCC TCA GCC TAC TAT AAT GCC-3'
P11: 5'-CTA GGG ATC CCG ACA GAT GCA CTA TTA GGC-3'

The PCR amplification mixture (50 µl volume) contained 1.25 units of Taq polymerase, 1.5 Mm $MgCl_2$, 50mM KCl, 10mM Tris-HCl (pH 8.3), 200 µM each of dATP, dGTP, dCTP, dTTP (Fermentas, Glen Burnie, MD, USA) 10 pmol of primers (p55 and p34) and 5µl of the extracted DNA. The second set of primers (p10 and p11) replaced the first set in the second PCR. For both PCR runs, the amplification protocol consisted of denaturation of template at 94^0C for 30 sec, annealing at 57^0C for 2 min followed by extension at 70^0C for 2 min for 35 cycles in a thermal cycler (MJ Research, Waltham, MA, USA). The PCR product was visualized using a gel documentation system (GelDoc XR, BioRad Inc, Hercules, CA, USA) after electrophoresis in 1.5% agarose gel containing ethidium bromide (0.5µg/ml).

Statistical analysis

In the absence of a gold standard test, the three tests were evaluated by latent class analysis (LCA) using latent class software Version 3.0 [Courtesy Dr. S.D. Walter, McMaster University, Canada]. Using the expectation-maximization algorithm the sensitivity and specificity was determined for each of the three tests used in addition to the prevalence. The analysis was performed as described by Sathish *et al*.[17]

Results

IgM antibodies to the 56 kDa antigen were detected in 51 of the 87 samples by ELISA. Weil-Felix (WF) test was positive in 25 samples and the 56 kDa antigen gene was amplified by nested PCR in 22 cases. Table 1 gives the number of samples positive in a single or a combination of tests.

By LCA [Table 2], IgM ELISA had a sensitivity of 100% and a specificity of 73%. The sensitivity of the Weil-Felix test and the PCR was significantly lower. The specificity of PCR was 100%, and that of the Weil-Felix (WF) test 94%. The prevalence was found to be 43% in our study population by LCA.

Interestingly, the 22 patients confirmed by PCR were also positive by IgM ELISA. IgM ELISA for scrub typhus was positive in all the eight patients who had an eschar, whereas PCR was positive only in six. In three ELISA-positive, but PCR-negative individuals, other causes of infection were detected and these are considered as false positive results for scrub typhus by IgM ELISA. Malaria was diagnosed in two individuals, peripheral smear showed *P. falciparum* in one while the other had *P. vivax*. The third case had pulmonary tuberculosis as acid-fast bacilli (AFB) were demonstrated in the sputum of this individual. In contrast, no false positive results were seen with PCR.

One target PCR product was sequenced to confirm the

Table 1: Description of results obtained by tests used in scrub typhus diagnosis (n=87)

Positive status category	Number of samples (%)
All positive	13 (15.0)
Weil-Felix and IgM ELISA positive	10 (11.5)
IgM ELISA and PCR positive	9 (10.3)
Weil-Felix only positive	2 (2.3)
IgM ELISA only positive	19 (21.8)
All negative	34 (39.1)

Table 2: LCA analysis: Prevalence, sensitivity, specificity of the three tests use

Test done	Prevalence (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
Weil-Felix test		0.59 (0.48, 0.69)	0.94 (0.86,0.98)
IgM ELISA	0.43 (0.33,0.54)	1.00 (0.95,0.99)	0.73 (0.62,0.82)
Nested PCR (56 kDa)		0.58 (0.47,0.68)	1.00 (0.95,0.99)

gene. The sequence was analyzed using BLAST (available from www.ncbi.nlm.nih.gov/BLAST). There was 99% homology with the 56 kDa gene of *O. tsutsugamushi* strains Taitung-2 and TW461, 97% homology with strain Hualien-1 and 95% homology with strains UAP6, HSB3, 405S, Iwataki-1, LP-1, Ikeda, and Yonchon. The GenBank accession number assigned to our sequence is GQ260638.

Discussion

Rapid diagnosis of scrub typhus is essential to reduce morbidity and mortality. Nested PCR is described as a very sensitive technique to detect *O. tsutsugamushi* DNA, making it a useful tool for early diagnosis of scrub typhus.[8] PCR to detect the gene encoding 56 kDa antigen has been used by most workers as this gene is found only in *O. tsutsugamushi* and variations in the same are responsible for the antigenic and genetic diversity of *O. tsutsugamushi*.[18]

In 2006, Sonthayanon and co-workers reported a sensitivity of 29% and 45% for the detection of 56 kDa antigen gene and the 16S rRNA gene respectively, using EDTA blood as the source of DNA. The overall specificity for both assays was 99% using the immunofluorescence assay (IFA)as the reference standard.[9] Fournier *et al.*, detected a 372 bp fragment of the 56 kDa gene in the buffy coat of only 10 of the 195 serologically confirmed cases of scrub typhus using a non-nested protocol.[18] Using DNA extracted from the buffy coat and using the same protocol including primers as used in the current study, Kim *et al.*, showed sensitivity and specificity of 82% and 100% respectively.[8]

Nested PCR using the primers used in our study is reported to be very sensitive as it detects 100 fg or two copies of *O. tsutsugamushi*.[7] However, we obtained a lower sensitivity as compared to IgM ELISA. UV spectrophotometry (ND-1000 spectrophotometer, Nanodrop Technologies, Inc. Wilmington, USA) confirmed the quality of extracted DNA to be good, thus ruling out technical error in DNA extraction. The low yield in PCR could be due to the presence of heme, a known inhibitor of PCR in the sample.[19] The improved sensitivity obtained by Kim *et al.*, also could be due to the volumes (50 µl) used for extracting DNA,[8] in contrast to 200 µl used by us, as recommended by the manufacturer. The high rate of false-negative PCR results for the patients whose IgM antibody titres were high might have also been caused by the clearance of bacteria by the immune system.[8] Though eschar is considered a better specimen as compared to blood clot, it is not found in all cases with scrub typhus. Hence, buffy coat is probably a more practical and suitable alternative for improving detection by nested PCR.[20]

Multiplex real-time PCR assays such as the one described by Prakash *et al.*,[21] which detect DNA of not only the scrub typhus agent but also those of agents responsible for spotted fever and typhus fever, need to be evaluated. This assay exhibits exquisite sensitivity, detecting even two copies/µl. The assay described by Paris *et al.*, on the other hand detects only 24 copies/µl of the 47 kDa gene of *O. tsutsugamushi*.[22]

Although Weil-Felix test had a high specificity, sensitivity was low for scrub typhus diagnosis, as reported earlier.[5] This calls for upgrading facilities and expertise for diagnosing scrub typhus in different parts of India, especially considering that the prevalence of this infection is very high among patients with acute onset fever. Until such time, the Weil-Felix test is likely to remain the only test available in most parts of India.

In conclusion, the good specificity and low sensitivity found in this evaluation of nested PCR using blood clot as sample suggests the need for further evaluations of this test using samples such as eschar and buffy coat. IgM ELISA is very sensitive but the Weil-Felix test is still useful for the diagnosis of scrub typhus in resource-poor settings.

References

1. Watt G, Parola P. Scrub typhus and tropical rickettsioses. Curr Opin Infect Dis 2003;16:429-36.
2. Kovacova E, Kazar J. Rickettsial diseases and their serological diagnosis. Clin Lab 2000;46:239-45.
3. Mathai E, Rolain JM, Varghese GM, Abraham OC, Mathai D, Mathai M, *et al*. Outbreak of scrub typhus in southern India during the cooler months. Ann N Y Acad Sci 2003;990:359-4.
4. Coleman RE, Sangkasuwan V, Suwanabun N, Eamsila C, Mungviriya S, Devine P, *et al*. Comparative evaluation of selected diagnostic assays for the detection of IgG and IgM antibody to *Orientia tsutsugamushi* in Thailand. Am J Trop Med Hyg 2002;67:497-503.
5. Prakash JA, Abraham OC, Mathai E. Evaluation of tests for serological diagnosis of scrubtyphus. Trop Doct 2006;36: 212-3.
6. Furuya Y, Yoshida Y, Katayama T, Kawamuri F, Yamamoto

S, Ohashi N, *et al*. Specific amplification of *Rickettsia tsutsugamushi* DNA from clinical specimens by polymerase chain reaction. J Clin Microbiol 1993;29:2628-30.
7. Saisongkorh W, Chenchittikul M, Silpapojakul K. Evaluation of nested PCR for the diagnosis of scrub typhus among patients with acute pyrexia of unknown origin. Trans Soc Trop Med Hyg 2004;98:360-6.
8. Kim DM, Yun NR, Yang TY, Lee JH, Yang JT, Shim SK, *et al*. Usefulness of nested PCR for the diagnosis of scrub typhus in clinical practice: A prospective study. Am J Trop Med Hyg 2006;75:542-5.
9. Sonthayanon P, Chierakul W, Wuthiekanun V, Blacksell SD, Pimda K, Suputtamongkol Y, *et al*. Rapid diagnosis of scrub typhus in rural Thailand using polymerase chain reaction. Am J Trop Med Hyg 2006;75:1099-102.
10. Lee SH, Kim DM, Cho YS, Yoon SH, Shim SK. Usefulness of eschar PCR for diagnosis of scrub typhus. J Clin Microbiol 2006;44:1169-71.
11. Kim DM, Kim HL, Park CY, Yang TY, Lee JH, Yang JT, *et al*. Clinical usefulness of eschar polymerase chain reaction for the diagnosis of scrub typhus: A prospective study. Clin Infect Dis 2006;43:1296-300.
12. Mahajan SK, Rolain JM, Kashyap R, Bakshi D, Sharma V, Prasher BS, *et al*. Scrub typhus in Himalayas. Emerg Infect Dis 2006;12:1590-2.
13. Jiang J, Chan TC, Temenak JJ, Dasch GA, Ching WM, Richards AL. Development of a quantitative real-time polymerase chain reaction assay specific for *Orientia tsutsugamushi*. Am J Trop Med Hyg 2004;70:351-6.
14. Sonthayanon P, Chierakul W, Wuthiekanun V, Phimda K, Pukrittayakamee S, Day NP, *et al*. Association of high *Orientia tsutsugamushi* DNA loads with disease of greater severity in adults with scrub typhus. J Clin Microbiol 2009;47:430-4.
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;104:63-71.
16. Paris DH, Aukkanit N, Jenjaroen K, Blacksell SD, Day NP. A highly sensitive quantitative real-time PCR assay based on the groEL gene of contemporary Thai strains of *Orientiat sutsugamushi*. Clin Microbiol Infect 2009;15:488-95
17. Sathish N, Manayani DJ, Shankar V, Abraham M, Nithyanandam G, Sridharan G. Comparison of IgM capture ELISA with a commercial rapid immunochromatographic card test and IgM microwell ELISA for the detection of antibodies to dengue viruses. Indian J Med Res 2002;115:31-6.
18. Fournier PE, Siritantikorn S, Rolain JM, Suputtamongkol Y, Hoontrakul S, Charoenwat S, *et al*. Detection of new genotypes of *Orientia tsutsugamushi* infecting humans in Thailand. Clin Microbiol Infect 2008;14:168-73.
19. Liu YX, Cao WC, Gao Y, Zhang JL, Yang ZQ, Zhao ZT, *et al*. *Orientia tsutsugamushi* in eschars from scrub typhus patients. Emerg Infect Dis 2006;12:1109-12.
20. Koh GC, Maude RJ, Paris DH, Newton PN, Blacksell SD. Diagnosis of scrub typhus. Am J Trop Med Hyg 2010;82: 368-70.
21. Prakash JA, Reller ME, Barat N, Dumler JS. Assessment of a quantitative multiplex 5' nuclease real-time PCR for spotted fever and typhus group rickettsioses and *Orientia tsutsugamushi*. Clin Microbiol Infect 2009;15:292-3.
22. Paris DH, Blacksell SD, Stenos J, Graves SR, Unsworth NB, Phetsouvanh R, *et al*. Real-time multiplex PCR assay for detection and differentiation of rickettsiae and orientiae. Trans R Soc Trop Med Hyg 2008;102:186-93.

Source of Support: Institutional review board, Christian Medical College, Vellore, India.,
Conflict of Interest: None declared.