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Serodiagnosis and Molecular Characterization of Scrub Typhus in and around Vijayapura, North Karnataka Region

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Abstract

Background: Scrub typhus or tsutsugamushi disease, transmitted by the bites of infected immature mites (chiggers) is a most covert re-emerging febrile infection currently. The disease is unnoticed or misdiagnosed due to low manifestation and lack of specific diagnostic tests at all levels. Failure of timely diagnosis leads to significant morbidity and mortality. Geographically this disease is widely endemic in a confined area of the Asia-Pacific region. In India, Scrub typhus infection is increasing and reported from various geographical parts during the past 10-15 yrs. Serological test is widely used for the diagnosis of the disease.

Aim & Objectives: To investigate the presence of Scrub typhus infection in and around Vijayapura of North Karnataka region using serological tests and nested PCR.

Materials and Method: During the period of 2015-17, a total of 209 patients presenting with acute febrile illness with rashes, body ache were examined for Scrub typhus infections by Weil Felix agglutination test and IgM ELISA. Further all the ELISA positive sampleswere tested by nested PCR using the specific primers of the gene encoding the immunodominant 56 kDa protein and PCR products were sequenced.

Results: Out of 209 cases, 39 (18.5%) samples showed agglutinationin Weil Felix antigen OXK test with titre 1:160 and above. In Scrub typhus IgM ELISA 13 (6.3%) samples were positive with the OD value more than 0.5. In nested PCR, two samples were amplified a 483 base pairs diagnostic segment of the gene encoding the 56 kDa antigen of *O. tsutsugamushi* using specific primers. PCR product was sequenced bidirectionally, and nucleotide sequences queried against NCBI BLAST programme to identify the sequenced sample.

Conclusion: Findings of our study demonstrated that, though more seropositivity of Spotted Fever Group Rickettsial infection is observed by WF test, scrub typhus infection is also circulating and causing acute febrile illness in and around Vijayapura, North Karnataka region. As the routinely used Weil felix test is less sensitive, inclusion of more specific tests like ELISA and nested PCR is very useful in proper diagnosis and patient management.

Keywords: *Weil Felix test, Rickettsia, Scrub typhus, ELISA, Nested PCR.*

Introduction

Rickettsial diseases are the most covert re-emerging febrile infections of the current era and mostly go unnoticed or misdiagnosed due to low manifestation and lack of specific diagnostic tests at all levels. Biogroups of Rickettsiaceae includes Spotted fever group, Typhus group and Scrub typhus[1]. Scrub typhus or tsutsugamushi disease is caused by the bacterium Orientia *tsutsugamushi*, which is transmitted by the

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bites of infected, immature mites (chiggers) belonging to the family Trombiculidae. Illness typically begins after the bite of an infected chigger and lasts for 7-10 days. The most common symptoms of infected persons are consistent fever of more than 7 days, headache, myalgia, rashes, eschar and lymphadenopathy. Delay in diagnosis and treatment leads to severe complications like central nervous system problems or circulatory collapse caused by disseminated intravascular coagulation and even death of the individual[2,3]. Geographically this disease is widely endemic in a confined area of the Asia- Pacific region, distributed in tsutsugamushi-triangle i.e. India and Nepal in the west; China, Japan, South Korea, and Taiwan in the north, and Australia and Indonesia in the south[2,3,4]. In India, the presence of Rickettsiosis has been reported from Jammu and Kashmir, Himachal Pradesh, Delhi, Rajasthan, Uttaranchal, West Bengal, Assam, Meghalaya, Karnataka, Andra Pradesh, Maharashtra, Tamil Nadu, Puducherry and Kerala[3-14].

During the last 10 - 15 years, several researchers from different geographical parts of India have reported seropositivity of scrub typhus infections using single or combination of serology based tests like, ELISA, Weil felix and Microimmunofluorescence assay etc[3-14]. Further, a few researchers have also demonstrated the PCR, a molecular test for the diagnosis of scrub typhus by detecting different genes, namely *56kDa*[15-20], *Groel*, and *16SrRNA*[16] from blood samples [15-19] and rodents [20].

When we reviewed the hospital medical records of last two years, we observed significantly high number of acute febrile illness cases diagnosed as Rickettsial infection on the basis of single serological test i.e. Weil felix. Though Scrub typhus infection has been increasing and reported from various geographical parts of India, no confirmatory data is available from north Karnataka region. Hence, we undertook this studyduring the period 2015– 2017 to investigate the existence of Scrub typhus infection in and around Vijayapur, Karnataka using both serological tests and molecular test- nested PCR.

Materials and Method

During the period of 2015-17, a total of 209 patients presenting with acute febrile illness, rashes and body ache were screened for the presence of Scrub typhus infection. It was a prospective study and the study group comprised of patients of all age-groups and both sexes who attended and admitted toOP/IP departments Shri B.M. Patil Medical College, Hospital and Research centre, Vijayapur and District hospital, Vijayapur. The patients with acute febrile illness, and already diagnosed as other infections like malaria, enteric fever, dengue during the sample collection were excluded from the study.

After obtaining the informed consent, 5 ml of Venus blood samples were collected from patients in plain tube and serum was separated and blood clots of all the samples were stored at -20°C.

Serological Tests

Weil Felix test: Serum samples were screened for the presence of antibodies against Scrub typhus infection by Weil felix tube agglutination test (Tulip diagnostics, Goa), in which the antibodies present in the serum reacts with antigens derived from various proteus species and exhibit agglutination. Samples with titres of 1:160and above for OXK were considered as positive for Scrub typhus infection.

IgM ELISA: All the samples were screened for the serological confirmation of the presence of specific antibodies against Scrub typhus infection by IgM ELISA (Inbios International, USA), a method which is reported to be more sensitive and specific by several researchers [3,4,8,10,11]. Both positive and negative controls provided with the kit were also included in the assay. Both the serological tests were performed strictly according to the manufacturer's instructions.

Nested PCR: Blood clot was homogenized and the DNA was extracted from 200 µl of homogenized blood using the QIAmp blood mini kit (Qiagen) as per manufacturer′s instructions. The eluted DNA was aliquoted and stored at -30ºC. Nested PCR was performed using two sets of primers to amplify a 483 base pairs segment of the gene encoding the 56 kDa antigen of *O. tsutsugamushi* as described by Furuya *et al*. (1993). The primers were procured from Integrated DNA Technologies (IDT).

Details of primers used are:

First set (Outer primer); P34: 5′-TCA AGC TTA TTG CTA GTG CAA TGT CTGC- 3′ and P55: 5′ -AGG GAT CCC TGC TGC TGT GCT TGC TGCG-3′

Second set (Inner primer); P10: 5′-GAT CAA GCT TCC TCA GCC TAC TAT AAT GCC-3′ and P11: 5′-CTA GGG ATC CCG ACA GAT GCA CTA TTA GGC-3′

Premixed ready to use green master mix (Promega, USA) was used for amplification, which consists of Taq Polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. Total volume of reaction mixture including templates was 50 µl. First PCR was performed with the template DNA using first set of primers (p34 & p55). The first PCR product was used as template for the second PCR with second set of primers (p10 & p11). Amplification protocol for both PCR was: initial denaturation of template at 95°C for 10 min, denaturation at 95°C for 30 sec,annealing at 55°C for 1min, extension at 70°Cfor 1 min for 35 cycles followed by final elongation at 70°C for 10 min in a thermal cycler (Aeris, ESCO). The amplicons were electrophoresed in a 1% agarose gel containing ethidium bromide (0.5µg/ml) and visualized in Gel documentation system system (Syngene, USA). PCR products were sequenced by Sanger sequencing method to identify the species.

Results

Out of 209 samples, 39 samples showed agglutination with OXK antigen with titre of 1:160 and above, and13 samples were positive by IgM ELISA. Interpretation was done as per the manufacturer instructions (Table 1).

Table 1: Results of comparison of two serological tests performed for the detection of Scrub typhus infection (n = 209).

Serological Tests	Total positive	IgM ELISA Positive	IgM ELISA Negative
Weil felix OXK positive	39	11	28
Weil felix OXK negative	170	02	168

Negatives include samples with titre 1:80 and below for OXK, and samples positive for other WF antigens except OXK. Samples non reactive with WF antigens were not evaluated.

In nested PCR, out of 13 ELISA positives, two samples amplified the gene encoding the 56 kDa antigen of O. *tsutsugamushi*. (Fig 1). One positive control received from Dept of Microbiology, BMCRI, Bangalore and one negative control was also tested along with the samples.

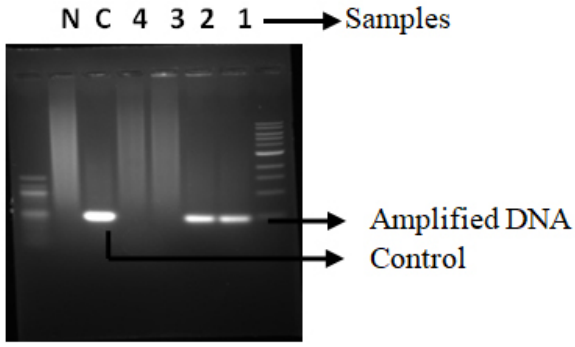

Fig 1. Agarose gel electrophoresis picture of amplified DNA by nested PCR. Lane 1- sample 1, lane 2- sample 2, lane 3 sample 3, lane 4 sample 4, lane -C positive control and lane - N negative control.

PCR product was sequenced bidirectionally using Applied Biosystems model 3730xl/ 3730XLPA-19137-017 at Eurofins genomics India Pvt. Ltd, and quality of sequences was checked with Finch TV Version 1.4 and consensus sequence was generated using Bio Edit software version 7.2. Further nucleotide sequences were queried against NCBI BLAST programme to identify the sequenced sample. NCBI BLAST program (blastn suite) showed 97 to 99% homology with the strains reported from KMC Manipal (MG283201.1), Andaman and Nicobar (MF457892.1), Pondicherry (KT970967.1) and Himalayan region (DQ286233.1), Uttar Pradesh (KR706188.1).

Discussion

Rickettsial infections are re-emerging in India and will lead to significant morbidity and mortality if failed to diagnose timely and take appropriate treatment. The diagnosis of scrub typhus is generally made by the history and clinical presentation. The vast variability and common clinical manifestations of the disease which is similar to other febrile illnesses makes the clinical diagnosis challenging. During the last decade, several researchers have reported the existence of rickettsial infection in different part of India using single or a combination of serological tests like Weil felix, ELISA and Microimmunofluorescence assay [1, 3-14] and using both Serological test and molecular test- PCR[16-19].

When we reviewed the last two years medical records of tertiary care facility Shri B.M. Patil medical college Hospital and research centre Vijayapura, we found that significant numbers of cases have been diagnosed using Weil felix test alone, and in which seropositivity for

SFG Rickettsiae or Indian Tick typhus was commonly reported than Scrub typhus. We also have observed and reported similar results in our previous serological study on R. conorii. In that study, out of 231 cases screened, 105 cases were positive for OX2 antigen by Weil felix test suggestive of R. *conorii* and 27 cases were confirmed serologically by IgM ELISA.

Though scrub typhus infection have been increasing and reported from various parts of India, Scrub typhus was not evaluated and no confirmatory data is available from north Karnataka region. Hence, we undertook this study to investigate the existence of Scrub typhus in Vijayapura area of Karnataka using both serological and molecular tests. All the cases included in the study were clinically suspected with fever of more than one week (100%), body ache (23%)and rashes (19%). We could not find eschar in any cases.

Out of 209 symptomatic cases screened, 39 (18.5%) were positive by Weil felix with titre of OXK >1:160 and 13 were positive by IgM ELISA suggestive of Scrub typhus infection. It is highly significant in this region even though the results obtained in our study shows considerable difference in percentage of positive cases reported by several researchers from different geographical parts of India like Karnataka [9,10], Andrapradesh[3, 13, 17], Pondicherry [8,16], Tamil Nadu[4,5,6,17], Himachal Pradesh[7,11,12] and Delhi [14]. We have observed more seropositivity during cooler months between August to January, and same was reported by few of the investigators [3,5,6].

Since both the serological tests exhibit significantly varied sensitivity and specificity according to the geographical area[3-13], the detection of causative organism directly from the patients sample would be highly important. Different PCR techniques have been reported during the last decade for detection of different genes of scrub typhus namely *56kDa*[15-20], *Groel* and *16SrRNA*[16] from blood samples [15-19] and rodents [20]. To substantiate the seropositivity observed in the study area, we performed the nested PCR on blood clots of ELISA positive samples for the confirmation of organism using the primers encoding the 56kDa gene of O. *tsutsugumushi* as described by *Furuya et.al*.[15]. While ELISA positive samples had high OD value, we succeeded to amplify a 483 bp segment of the gene encoding the 56 kDa antigen of *O. tsutsugamushi* in only two samples which were positive by both the serological tests Weil Felix and IgM ELISA. Results of Nucleotide sequences queried against NCBI BLAST programme showed 97 to 99% homology with the strains reported from KMC Manipal (MG283201.1), Andaman and Nicobar (MF457892.1), Pondicherry (KT970967.1), Himalayan region (DQ286233.1) and Uttar Pradesh (KR706188.1).

Conclusion

Findings of our study demonstrated that though more seropositivity of SFG Rickettsial infection is observed by Weil Felix test, scrub typhus infection is also circulating and causing acute febrile illness in and around Vijayapura, North Karnataka region. As the routinely used Weil felix test is less sensitive, inclusion of specific tests like ELISA and nested PCR is very useful in proper diagnosis and patient management.

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Ethical Clearence: Ethical clearance was obtained from ethical committee, BLDE University Vijayapur.

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