Molecular confirmation & characterization of *Rickettsia conorii* in north India: A report of three cases

Manisha Biswal¹, Kamran Zaman¹,†, Vikas Suri², Srikanth Gopi², Abhay Kumar¹, T. Gopi¹, Shashi Vig¹, Navneet Sharma² & Ashish Bhalla²

Departments of ¹Medical Microbiology & ²Internal Medicine, Postgraduate Institute of Medical Education & Research, Chandigarh, India

Received January 12, 2018

**Background & objectives:** In India, spotted fever group rickettsiae (SFGR) are an underdiagnosed cause of acute febrile illness (AFI). The non-specific Weil-Felix test is the first diagnostic modality for the diagnosis of SFGR in many laboratories due to the lack of advanced diagnostic facilities in developing countries. The aim of this study was to detect SFGR using molecular methods in the patients, presenting with AFI in a tertiary care centre in north India.

**Methods:** Consecutive patients (>14 yr of age) with AFI were enrolled over a six month period. Standard investigations for common pathogens causing AFI in India (malaria, dengue, scrub typhus, leptospirosis and enteric fever) were carried out. In patients who were negative for all of the above investigations, blood was subjected to polymerase chain reaction (PCR) targeting outer membrane protein A (*ompA*) gene of *Rickettsia*.

**Results:** Of the 51 patients with an undiagnosed aetiology, three were positive by *ompA* PCR. Two of the PCR products produced good sequences and BLAST identification confirmed them as *Rickettsia conorii*. The sequences of *R. conorii* reported from south India clustered with two previously reported novel rickettsial genotypes. The study sequences clustered in a group different from that of *Rickettsia* spp. of the south Indian sequences reported earlier.

**Interpretation & conclusions:** This study showed the existence of *R. conorii* in north India. Testing for SFGR may be included in the diagnostic workup of AFI for better disease management.

**Key words** Acute febrile illness - India - *Rickettsia conorii* - rickettsial infection - spotted fever

*Rickettsia conorii*-mediated spotted fever may be an underdiagnosed cause of acute febrile illness (AFI) in India, as it has been sporadically reported from this geographical region¹⁻³. The mortality rate in spotted fever is variable but can be high if there is a delay in the diagnosis and treatment¹,⁴,⁵. Most studies on the prevalence of spotted fever group rickettsioses (SFGR) in India are based largely on serological tests such as Weil-Felix⁶,⁷, ELISA⁶,⁸,⁹ and immunofluorescence assay¹⁰. The serological diagnosis has limitations and accurate disease correlation can be made only by DNA detection or by

¹Present address: ICMR-Regional Medical Research Centre, Gorakhpur, Uttar Pradesh, India

© 2020 Indian Journal of Medical Research, published by Wolters Kluwer - Medknow for Director-General, Indian Council of Medical Research
culture. However, the culture of the organism requires biosafety level 3 (BSL-3) containment facilities and is restricted to reference laboratories\(^1\). In India, the prevalence of \(R.\ conorii\) in febrile patients has been evaluated with the help of polymerase chain reaction (PCR) and sequencing in a study from south India\(^11\). A novel spotted fever \(Rickettsia\) was detected in a Japanese traveller returning from India\(^12\). Though the tick vector, \(Rhipicephalus sanguineus\) sensu lato, has been found in 21 States in India\(^13\), there are many gaps in the knowledge about the true burden of this infection in India. The aim of the present study was to detect the presence of SFGR in patients presenting with AFI in a tertiary care hospital in north India using molecular diagnosis.

**Material & Methods**

Consecutive patients above the age of 14 yr presenting with AFI to the Emergency Medical Ward of Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, over a six-month period (June-November, 2014) were enrolled in the study. A detailed clinical history was noted. The patients were thoroughly examined for skin rash, eschar and manifestations of bleeding. The standard workup for fever, which included peripheral blood smear and antigen testing for malarial parasites, blood culture by BACTEC, Widal test for enteric fever, NS1 antigen and/or IgM ELISA for dengue, PCR and/or IgM ELISA for scrub typhus and ELISA and/or microscopic agglutination test (MAT) for leptospirosis was performed in the department of Medical Microbiology. In patients negative for all of the above infections, PCR was carried out for the detection of the outer membrane protein \(A\) (\(ompA\)) gene of \(Rickettsia\). Primers described by Regnery et al\(^4\) were employed in this study for amplification purpose and the amplification was carried out as described. Nuclease-free water was used as negative control and DNA of \(R.\ conorii\) Malish strain (gifted from Prof. Pierre-Edouard Fournier, URMITE, Marseille, France) was used as the positive control. All measures were taken to avoid cross-contamination during the PCR processing. The amplicons were subjected to gel electrophoresis and band patterns visualized. The purified amplicons were subjected to DNA sequencing using BigDye Terminator Cycle Sequencing (Applied Biosystems, USA). The DNA sequences obtained were subjected to BLAST search (http://blast.ncbi.nlm.nih.gov/blast) to identify the agent. The phylogenetic tree was constructed using MEGA version 7\(^15\). The evolutionary history was constructed using the neighbour-Joining method, and evolutionary distance matrix was computed using the Maximum Composite Likelihood method\(^15\).

The protocol was approved by the Institutional Ethics Committee (ECC reference no. NK/1300/MD/1261), PGIMER, Chandigarh. Written informed consent was obtained from all the patients.

**Results**

A total of 135 patients diagnosed with an acute undifferentiated febrile illness, who presented to the emergency medical ward for adults were enrolled in this study. The most common diagnosis was scrub typhus seen in 54 (40\%) patients followed by malaria in 13 (9.6\%) patients and in 51 patients (37.8\%) no definite diagnosis was established. These 51 patients were tested for the presence of \(R.\ conorii\) DNA by \(ompA\) PCR; among them three patients turned out to be positive. All three patients were young male, one each hailing from the States of Punjab, Haryana and Himachal Pradesh. All presented with fever with non-specific symptoms. None had a history of travel and no history of a bite by an arthropod (tick). Rash or eschar could not be found in any of these patients diagnosed with \(R.\ conorii\). The clinical symptoms and signs of the three \(R.\ conorii\) patients are shown in Table I.

| Amplicon Size | Sequence Similarity |
|---------------|---------------------|
| 500 bp        | 100\%               |
| 1000 bp       | 99\%                |
| 1500 bp       | 98\%                |

Of the three \(ompA\)-positive amplicons with same band size, only two produced good sequences and BLAST identification confirmed these as \(R.\ conorii\). On the basis of same amplicon size and in view of that all measures were taken to avoid cross-contamination during the PCR processing, the third amplicon was also considered similar to other two sequences. Both sequences were submitted to the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/), and were assigned the accession numbers PGI_RC1_KX016792 and PGI_RC2_KX016793, respectively. The PGI_RC1 \(ompA\) sequence showed 100 per cent similarity to \(R.\ conorii\) clone 09 (KR401144) and PGI_RC2 showed 100 per cent similarity to \(R.\ conorii\) subsp. \(conorii\) clone 45(JN182802). A phylogenetic tree constructed by the neighbour-joining algorithm using MEGA7 to compare all the sequences of \(R.\ conorii\) reported in India and other parts of the world. The sequences from these patients, grouped in a cluster consisting of \(R.\ conorii\) and \(R.\ conorii\) subsp. \(conorii\) (Figure). The sequences of \(R.\ conorii\) reported from south India, \(Rickettsia\) sp. CMC MICRO 1-4 (GenBank accession...
Table I. Clinical and laboratory features of three *Rickettsia conorii*-positive patients

| Characteristics                  | Patient 1                  | Patient 2                  | Patient 3                  |
|----------------------------------|---------------------------|---------------------------|---------------------------|
| Gender                           | Male                      | Male                      | Male                      |
| Age (yr)                         | 14                        | 36                        | 24                        |
| Location                         | Panchkula, Haryana        | Solan, Himachal Pradesh   | Nawanshahr, Punjab        |
| Fever                            | +                         | +                         | +                         |
| Duration of fever (days)         | 14                        | 1                         | 10                        |
| Headache                         | -                         | +                         | -                         |
| Cough                            | -                         | +                         | -                         |
| Shortness of breath              | -                         | +                         | -                         |
| Rash/eschar/petechia             | -                         | -                         | -                         |
| Jaundice                         | -                         | +                         | -                         |
| Myalgia                          | -                         | +                         | -                         |
| Bleeding manifestations          | -                         | -                         | -                         |
| Hepatomegaly                     | +                         | +                         | -                         |
| Splenomegaly                     | -                         | -                         | -                         |
| Chest X-ray                      | Mild right pleural effusion | Bilateral diffuse infiltrates | -                         |
| Abdominal ultrasonography       | Hepatomegaly, splenomegaly, mild ascites | Hepatomegaly | Normal |
| Haemoglobin (g/dl)               | 8.3                       | 10.1                      | 14                        |
| Total leucocytes count (cells per µl) | 7600                  | 12900                     | 6600                      |
| Platelet count (×10^3/µl)        | 50                        | 71                        | 55                        |
| Total bilirubin (mg/dl)          | 4.53                      | 5.6                       | 0.6                       |
| Conjugated bilirubin (mg/dl)     | 4.0                       | 1.2                       | 0.2                       |
| Total protein (g/dl)             | 5.48                      | 5.6                       | 6                         |
| Albumin (g/dl)                   | 2.7                       | 3.1                       | 3.4                       |
| Urea (mg/dl)                     | 30                        | 51                        | 24                        |
| Creatinine (mg/dl)               | 0.49                      | 1.7                       | 0.6                       |
| Other investigations             | EBV and CMV IgM - negative | -                         | -                         |

Organ dysfunction

| PaO2/FiO2                      | 410                       | 217                       | 412                       |
| ARDS                            | No                        | Yes                       | No                        |
| Hypotension                     | No                        | No                        | No                        |
| GCS                             | 15                        | 15                        | 15                        |
| SOFA score at admission         | 4                         | 7                         | 2                         |
| Therapy                         | Intravenous ceftriaxone 1 g twice daily (b.i.d) and oral doxycycline 100 mg b.i.d, given for total seven days | Intravenous ceftriaxone 1 g b.i.d and oral doxycycline 100 mg b.i.d, given for total seven days | Intravenous ceftriaxone 1 g b.i.d and oral doxycycline 100 mg b.i.d, given for total seven days |
| Outcome                         | Recovered                 | Recovered                 | Recovered                 |

PaO2/FiO2, ratio of partial pressure arterial oxygen and fraction of inspired oxygen; ARDS, acute respiratory distress syndrome; GCS, Glasgow coma scale; SOFA, sequential organ failure assessment; EBV, Epstein-Barr virus; CMV, cytomegalovirus

nos. HM587248-HM587251) clustered with two reported novel *Rickettsia* genotypes, *Candidatus Rickettsia kellyi* (DQ080005) and *Rickettsia* sp. Tenjiku01 (LC089865) from the south India. Our sequences clustered in the group completely different from that of *Rickettsia* sp. reported from the south India.
Indian studies\textsuperscript{11,12,16}. The distance matrix revealed an evolutionary divergence between sequences of north and south Indian isolates of \textit{R. conorii} (Table II).

**Discussion**

A total of 51 patients with AFI who were negative for the common causes of fever were studied. Rickettsial \textit{ompA} PCR detected three patients with spotted fever. There is a strong possibility that most of these infections go undiagnosed because of the low index of clinical suspicion due to the non-specific symptoms and lack of a suitable diagnostic test\textsuperscript{1}. None of our patients reported rash and eschar. The rash in case of spotted fever appears on 2-5 days after onset of symptoms and may be absent in approximately 9-10 per cent\textsuperscript{11,17-19}. Two of our patients presented after 10 days of fever. Eschar may not be present in all cases; most often, it is missed and masked by skin complexion in Indian patients\textsuperscript{11,18,20}. The clinical presentation and severity of these infections may differ geographically based on the hypothesis that the pathogenic potential of the infecting strains may differ. The \textit{Rickettsia} infections are more rampant during post-monsoon season as reported earlier\textsuperscript{8,21}. In the present study, all three patients presented during the post-monsoon season (July to September), and recovered completely after therapy with oral doxycycline. Doxycycline is the drug
of choice for spotted fever and is most effective when initiated within the first five days of illness, as early administration of doxycycline in adults and children can prevent severe illness and death\cite{22,23}. Azithromycin, when compared with other macrolides, is more effective in the case of spotted fever and can be used as an alternative. Azithromycin was shown to be ineffective in severe spotted fever patients\cite{24}. However, Colomba et al\cite{25} showed that azithromycin was the better choice for children with Mediterranean spotted fever.

The sequences obtained from our patients grouped into a cluster composed of \textit{R. conorii} subsp. \textit{conorii} and subsp. \textit{indica}. In the studies from south India\cite{11,16}, four \textit{Rickettsia} sp. sequences clustered with the earlier reported novel \textit{Rickettsia} genotypes \textit{Candidatus Rickettsia kellyi}. Our sequences clustered in the group different from that of \textit{Rickettsia} sp. reported in the different south Indian studies. This showed a diversity in the strains isolated from different parts of our country. Multicentric studies involving a large number of patients are required to elucidate the genetic diversity of all \textit{R. conorii} strains circulating in different parts of India.

In the present study, the identification was done based on the sequences obtained from the gene encoding surface proteins \textit{ompA}, which is known for its immunogenicity in humans due to its surface location\cite{26}. The limitation of our study was that only single gene (\textit{ompA}) was used instead of three different genes used for identifying the rickettsiae\cite{27}. However, several studies have shown \textit{ompA} to be more specific and capable of demonstrating marked diversity; thereby \textit{ompA} gene alone can serve as a potential tool for differentiating various SFG rickettsiae\cite{14,26,28,29}.

In our study, no serological assay for spotted fever was performed; however, ELISA for scrub typhus was done in all cases as the incidence of scrub typhus was higher in this region\cite{9,20}. Early initiation of appropriate antibiotic is important for the favourable outcome in spotted fevers caused by a \textit{Rickettsia}\cite{30,31}. Most of the patients respond well to antibiotics such as doxycycline, and somewhat less effectively to macrolides and chloramphenicol\cite{13,32}.

In conclusion, this study showed the existence of \textit{R. conorii} in north India. Tests for SFGR may be included in the diagnostic workup of AFI in north India also.

**Financial support & sponsorship:** None.

**Conflicts of Interest:** None.

**References**

1. Batra HV. Spotted fevers & typhus fever in Tamil Nadu. Indian J Med Res 2007; 126 : 101-3.
2. Mahajan SK, Kashyap R, Sankhyan N, Sharma V, Rolain JM, Prasher BS, et al. Spotted fever group rickettsioses in Himachal Pradesh. J Assoc Physicians India 2007; 55 : 868-70.
3. Rahi M, Gupte MD, Bhargava A, Varghese GM, Arora R. DHR-ICMR Guidelines for diagnosis management of rickettsial diseases in India. Indian J Med Res 2015; 141 : 417-22.
4. Chapman AS, Bakken JS, Folk SM, Paddock CD, Bloch KC, Krusell A, et al. Diagnosis and management of tickborne rickettsial diseases: Rocky Mountain spotted fever, ehrlichioses, and anaplasmosis-United States: A practical guide for physicians and other health-care and public health professionals. MMWR Recomm Rep 2006; 55 : 1-27.
5. Parola P, Paddock CD, Raoult D. Tick-borne rickettsioses around the world: Emerging diseases challenging old concepts. Clin Microbiol Rev 2005; 18 : 719-56.

6. Farhana A, Bai N, Kanth F, Farooq R, Haq IU, Shah P. Serological evidence of scrub typhus among cases of PUO in the Kashmir valley- A hospital based study. J Clin Diag Res 2016; 10 : DC24-6.

7. Kamarasu K, Malathi M, Rajagopal V, Subramani K, Jagadeeshramasamy D, Mathai E. Serological evidence for wide distribution of spotted fevers & typhus fever in Tamil Nadu. Indian J Med Res 2007; 126 : 128-30.

8. Kalal BS, Puranik P, Nagaraj S, Rego S, Shet A. Scrub typhus and spotted fever among hospitalised children in South India: Clinical profile and serological epidemiology. Indian J Med Microbiol 2016; 34 : 293-8.

9. Sethi S, Prasad A, Biswal M, Hallur VK, Mewara A, Gupta N, et al. Outbreak of scrub typhus in North India: A re-emerging epidemic. Trop Doct 2014; 44 : 156-9.

10. Khan SA, Bora T, Chattopadhyay S, Jiang J, Richards AL, Dutta P. Seroepidemiology of rickettsial infections in Northeast India. Trans R Soc Trop Med Hyg 2016; 110 : 487-94.

11. Prakash JA, Sohan Lal T, Rosemol V, Verghese VP, Pulimood SA, Heller M, et al. Molecular detection and analysis of spotted fever group Rickettsia in patients with fever and rash at a tertiary care centre in Tamil Nadu, India. Pathog Glob Health 2012; 106 : 40-5.

12. Takajo I, Sekizuka T, Fujita H, Kawano A, Kawaguchi T, Matsuda M, et al. Possible case of novel spotted fever group rickettsiosis in traveler returning to Japan from India. Emerg Infect Dis 2016; 22 : 1079-82.

13. Ghosh S, Nagar G. Problem of ticks and tick-borne diseases in India with special emphasis on progress in tick control research: A review. J Vector Borne Dis 2014; 51 : 259-70.

14. Regnery RL, Spruill CL, Plikaytis BD. Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. J Bacteriol 1991; 173 : 1576-89.

15. Kumar S, Stecher G, Tamura K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 2016; 33 : 1870-4.

16. Rolain JM, Mathai E, Lepidi H, Somashekar HR, Mathew LG, Prakash JA, et al. “Candidatus rickettsia kelleyi,” India. Emerg Infect Dis 2006; 12 : 483-5.

17. Mahajan SK. Rickettsial diseases. J Assoc Physicians India 2012; 60 : 37-44.

18. Rathi N, Rathi A. Rickettsial infections: Indian perspective. Indian Pediatr 2010; 47 : 157-64.

19. Sexton DJ, Corey GR. Rocky Mountain “spotless” and “almost spotless” fever: A wolf in sheep’s clothing. Clin Infect Dis 1992; 15 : 439-48.