Indian J Med Res 151, January 2020, pp 59-64
DOI: 10.4103/ijmr.IJMR_92_18

Quick Response Code:

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

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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 region[1-3]. The mortality rate in spotted fever is variable but can be high if there is a delay in the diagnosis and treatment[1,4,5]. Most studies on the prevalence of spotted fever group rickettsioses (SFGR) in India are based largely on serological tests such as Weil-Felix[6,7], ELISA[6,8,9] and immunofluorescence assay[10]. The serological diagnosis has limitations and accurate disease correlation can be made only by DNA detection or by

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culture. However, the culture of the organism requires biosafety level 3 (BSL-3) containment facilities and is restricted to reference laboratories[3]. 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*[14] 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. 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 ($\times 10^3$/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			
PaO_2/FiO_2	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

PaO_2/FiO_2, 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[11,12,16]. Our sequences clustered in the group completely different from that of *Rickettsia* sp. reported from the south

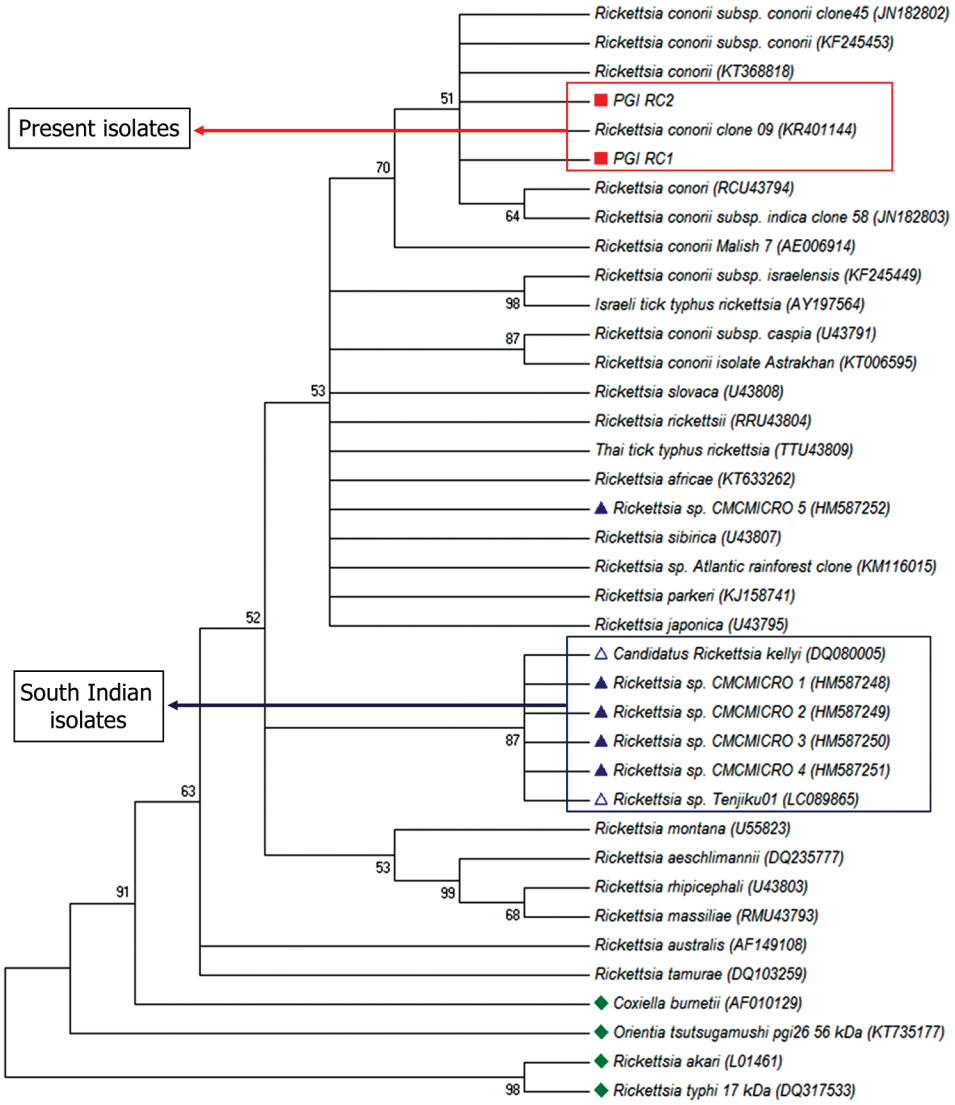

Figure. A phylogenetic tree constructed based on *ompA* gene using MEGA7 software. The sequences of *Coxiella burnetii* (AF010129), *Orientia tsutsugamushi* (735177), *Rickettsia akari* (L01461) and *Rickettsia typhi* (DQ317533) used as outgroups. Geometric shapes and colour indicate sequences from the present study and other Indian studies.

Indian studies[11,12,16]. The distance matrix revealed an evolutionary divergence between sequences of north and south Indian isolates of *R. conorii* (Table II).

Discussion

A total of 51 patients with AFI who were negative for the common causes of fever were studied. Rickettsial *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[3]. 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[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[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 *Rickettsia* infections are more rampant during post-monsoon season as reported earlier[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

Table II. Distance matrix showing the evolutionary divergence between sequences of Indian *Rickettsia conorii* isolates

Indian *R. conorii* isolates*	1	2	3	4	5	6	7	8	9	10
*Rickettsia_conorii*_clone_09_(KR401144)										
*Candidatus_Rickettsia_kellyi*_(DQ080005)	0.1033									
*Rickettsia*_sp._CMCMICRO_1_(HM587248)	0.1033	0.0000								
PGI_RC1_KX016792	0.0000	0.1033	0.1033							
PGI_RC2_KX016793	0.0000	0.1033	0.1033	0.0000						
*Rickettsia*_sp._CMCMICRO_2_(HM587249)	0.1033	0.0000	0.0000	0.1033	0.1033					
*Rickettsia*_sp._CMCMICRO_3_(HM587250)	0.1033	0.0000	0.0000	0.1033	0.1033	0.0000				
*Rickettsia*_sp._CMCMICRO_4_(HM587251)	0.1033	0.0000	0.0000	0.1033	0.1033	0.0000	0.0000			
*Rickettsia*_sp._CMCMICRO_5_(HM587252)	0.0458	0.1033	0.1033	0.0458	0.0458	0.1033	0.1033	0.1033		
*Rickettsia*_sp._Tenjiku01_(LC089865)	0.1033	0.0000	0.0000	0.1033	0.1033	0.0000	0.0000	0.0000	0.1033	

*The distance matrix shows the number of base substitutions per site between sequences after eliminating the gaps and missing data and analysis was conducted using the maximum composite likelihood model

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[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[24]. However, Colomba *et al*[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 *R. conorii* subsp. *conorii* and subsp. *indica*. In the studies from south India[11,16], four *Rickettsia* sp. sequences clustered with the earlier reported novel *Rickettsia* genotypes *Candidatus Rickettsia kellyi*. Our sequences clustered in the group different from that of *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 *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 *ompA*, which is known for its immunogenicity in humans due to its surface location[26]. The limitation of our study was that only single gene (*ompA*) was used instead of three different genes used for identifying the rickettsiae[27]. However, several studies have shown *ompA* to be more specific and capable of demonstrating marked diversity; thereby *ompA* gene alone can serve as a potential tool for differentiating various SFG rickettsiae[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[9,20]. Early initiation of appropriate antibiotic is important for the favourable outcome in spotted fevers caused by a *Rickettsia*[30,31]. Most of the patients respond well to antibiotics such as doxycycline, and somewhat less effectively to macrolides and chloramphenicol[3,32].

In conclusion, this study showed the existence of *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.

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