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Detection of acute Q fever human cases by indirect immunofluorescence & real-time polymerase chain reaction in a tertiary care hospital in Puducherry

Sir,

Coxiella burnetii, the causative agent of Q fever (QF) is reported across the world, with the exception of New Zealand and Antartica[1]. It is a potential agent of bioterrorism and classified under Category B pathogen, requiring Bio-safety Level 3 containments for handling isolation. The true prevalence of QF is unknown/underestimated in many countries including India[2]. Human acquires infection through domestic livestock by inhalation of infective aerosols liberated into the environment from aborted animals and in a few cases by consumption of unpasteurized milk[1]. The clinical presentation of acute QF varies from sub-clinical flu-like illness to pneumonia with fever, severe headache, myalgia, atypical pneumonia, hepatitis, joint and muscle pain[1-4]. Endocarditis is the most common manifestation of chronic QF[1,5]. Diagnosis of QF is through serological tests such as the complement fixation test, enzyme-linked immunosorbent assay (ELISA), immunofluorescence assay (IFA) and molecular tests for *C. burnetii* DNA[6-15]. Coxiellosis in domestic animals of India has been reported by several researchers[16-19].

The objective of our study was to detect QF in Puducherry and neighbouring districts of Tamil Nadu by IFA and real-time quantitative PCR (qPCR) in patients with acute febrile illness/pneumonitis of 1-15 days duration. This retrospective study was conducted during April 2014-July 2015 at Mahatma Gandhi Medical College and Research Institute, a tertiary care teaching hospital, Puducherry, after receiving approval from the Institutional Human Ethics Committee. Written informed consent was obtained from patients. Blood (3-5 ml) was collected in the sterile tube without anti-coagulant under aseptic conditions. Patients with fever/pneumonitis of 1-15 days duration, headache, myalgia, rash, hepatomegaly, splenomegaly, hepatitis, abdominal pain, nausea and vomiting were included for this study. Those with fever due to urinary tract infection/malaria/enteric fever; culture-positive bacterial pneumonia; patients with other bloodstream infections; bleeding disorders and fever for more than four weeks duration (pulmonary tuberculosis), were excluded.

A total of 76 patients were selected initially, but only 41 patients provided both acute and convalescent blood samples. Acute samples were collected when the patients had symptoms of 1-15 days duration. Convalescent samples were collected two to three weeks later. The serum was separated, aliquoted and kept frozen at −20ºC till the performance of IFA and qPCR. The interval between the collection of blood samples and testing for IFA and qPCR varied from one to five months. IFA was performed in batches and for QF qPCR, the samples were sent to Defence Research and Development Establishment (DRDE), Gwalior, where batch testing was carried out. IFA was carried out on acute as well as convalescent serum samples to detect IgM and IgG antibodies for both phase I and II. *C. burnetii* IFA (Fuller Laboratories, California, USA), QM-120 and QG-120 kits were employed, strictly adhering to the instructions of the manufacturers. Slides were scored as negative, 1+, 2+, 3+ and 4+. As per the kit, the significant titre in acute QF for phase II IgM was 1:16 and IgG 1:256. Regarding phase I IgM and IgG, the significant titres were ≥1:16 for both.

For qPCR, genomic DNA from serum samples was extracted using a DNeasy Tissue kit (Qiagen, Hilden, Germany). Extracted DNA samples were subjected to TaqMan real-time PCR, based on amplification of a 70 bp fragment of repetitive sequence *IS*1111 gene[15]. The PCR reactions were carried out in a StepOne Real-time PCR system (Applied Biosystems, USA) using TaqMan Universal Master Mix II (Invitrogen,

USA) according to the manufacturer's instructions. The results were analyzed with StepOne software v2.3 (Applied Biosystems)[3].

Statistical analysis of data was performed using Chi-square test with or without the Yates correction and Fisher's exact test. Student's *t* test was used to compare the days of fever at the time of collection of the first serum sample. QF positive and negative patients were also compared.

The patients included 20 children and 21 adults. Male and female ratio was 18:23. Patients' age ranged from 1-61 years. Mean age of patients was 20.41±16.97 yr with 95 per cent confidence interval (95% CI, 15.21-25.61). The number of days of fever at the time of collection of the first serum sample and mean with standard deviation calculated on patients with QF positive was 8±2.05 days and in QF negative was 8.31±4.08 days with 95 per cent CI of 7.07 - 8.92 and 6.6-10.0, respectively.

Paired serum samples of patients were subjected to IFA test against phase I and II IgM+IgG. The IFA titres ranged from ≥1:16 to 1:1024. Seroconversion was observed in only one patient (Sl. No. 16) against phase II IgG (1:16 à 1:128). Although, as per the kit, the significant titre for IgM phase II was 1:16 and IgG phase II 1:256, a cut-off value of 1:32 was selected for IgM phase II and ≥1:128 for IgG phase II to confirm acute QF, based on findings of earlier studies[1,6-12].

As outlined in Table I, 22 of 41 (Sl. No. 20-41) patients were negative by both IFA and qPCR. Among 19, four (Sl. No. 1-4) were negative by IFA, but positive by qPCR. Of the 15 IFA positive patients, only two showed PCR positivity and the remaining 13 were PCR negative.

Six patients (Sl. No. 1-4, 5 & 6) were considered to have acute QF due to the detection of *C. burnetii* DNA in their blood by qPCR, irrespective of IFA results. Their Ct (cycle threshold) values varied from 29.8 to 33.5. Besides, three patients (Sl. No. 7, 8 & 9) were confirmed by serology alone as acute QF, since they were positive for both phase II IgM and IgG with significant titres. The fifth patient (Sl. No. 5) showed titre of 1:64 for phase I IgG (significant) but not for phase II IgG (titre was only 1:64, whereas the significant titre was ≥1:128). The sixth patient was positive for only phase II IgM (1:32) hence considered seronegative, but

Table I. Results of Q fever immunofluroescence assay (QF IFA) immunoglobulin M, immunoglobulin G and quantitative polymerase chain reaction (qPCR) (n=41)

Serial No.	QF IFA assay				qPCR	Diagnosis	Animal contacts
	IgM		IgG				
	Phase I titres A/C	Phase II titres A/C	Phase I titres A/C	Phase II titres A/C			
1-4*	-/-	-/-	-/-	-/-	+	Acute QF	
5	-/-	-/-	64/64	64/64	+		Residence near abattoir
6	-/-	32/-	-/-	-/-	+		
7	-/-	32/512	-/-	256/256	-		
8#	64/128	64/128	1024/512	1024/1024	-		Sheep
9	-/-	512/64	128/256	256/512	-		
10	-/-	-/-	64/32	128/128	-	Probable acute QF/ past QF	
11	-/-	-/-	32/64	256/256	-		Goat
12	-/-	-/-	64/64	128/128	-		
13	-/-	-/-	64/128	128/256	-		
14	-/-	-/-	128/128	128/256	-		Sheep
15	-/-	-/-	-/-	64/128	-		
16	-/-	-/-	-/-	16/128	-		
17	-/-	-/-	-/-	128/128	-		
18-19	-/-	-/-	-/-	128/256	-		
20-41	-/-	-/-	-/-	-/-	-		Dog/cattle/goat/sheep$

#A QF, probably progressing to chronic QF, *One patient had pneumonitis, $Thirteen IFA negative patients had animal contacts.
A, acute; C, convalescent; IgM, immunoglobulin M; IgG, immunoglobulin G

with PCR positivity. Seventh patient (Sl. No. 7) had both phase II IgM and IgG antibodies and also showed 16-fold increase in titre in the paired serum samples in phase II IgM (1:32 → 1:512) and also positive for phase II IgG (1:256). Nineth patient (Sl. No. 9) had significant antibodies to phase II IgM, IgG and phase I IgG. In addition, eight-fold decrease was observed in titre between the acute and convalescent serum (1:512 → 1:64). Based on our findings, 10 patients (Sl. No. 10-19) came under the category of probable acute QF/past QF.

Based on the algorithm set by CDC[1] and Jager *et al*[9], regarding interpretation of QF IFA results, only five of these cases (Sl. No. 5-9) could be confirmed as acute QF and the remaining 10 (Sl. No. 10-19) (24.3%) as probable acute QF/resolved past QF, including one case of acute QF probably progressing to chronic QF (Sl. No. 8).

Table II details the clinical and laboratory parameters of *C. burnetii* antibody positive and negative patients. Most of these patients had common clinical features such as myalgia, chills and rigor, abdominal pain, nausea, vomiting and hepatomegaly. No significant differences were observed between the two categories except for splenomegaly (P<0.05). Five IFA positive (including two qPCR positive) and 13 IFA negative patients had a history of domestic animal contact such as cattle, sheep, goat, dog or their residence situated near the abattoir (Table I).

QF PCR is positive in the early stage of acute illness and tends to become negative when antibodies start appearing. The chronological order of appearance of antibodies in acute QF is as follows: IgM phase II → IgG phase II → IgM phase I → IgG phase I[15]. Phase II IgM antibodies are present in acute infection, whereas phase I antibodies are associated with chronic infections[1,5,8,12,13]. Detection of phase II IgG in both acute and convalescent samples and demonstration of seroconversion/four-fold increase in titre confirms a diagnosis of acute QF[8]. The presence of solitary phase II IgM in acute samples has little significance[11].

Laboratory definition and interpretation of acute QF[9] based on gold standard IFA is as follows: (*i*) IgM phase II + IgG phase II antibodies → acute QF; (*ii*) IgG phase II with or without IgG phase I → resolved past QF; and (*iii*) other combination *viz*., IgM/IgG phase II and/or IgM/IgG phase I → inconclusive, PCR to be performed.

Table II. Clinical and laboratory parameters of febrile patients (n=41)

Clinical/laboratory findings	QF positive (n=19)	QF negative (n=22)	Total (n=41)
Fever ≤14 days	19	22	41
Chills and rigor	12	14	26
Myalgia	13	8	21
Headache	8	8	16
Cough and expectoration	5	5	10
Abdominal pain	8	6	14
Hepatomegaly	3	5	8
Splenomegaly	7*	1	8
Malaise	2	4	6
Nausea	4	4	8
Vomiting	5	9	14
Pneumonitis	2	2	4
Lymphadenopathy	-	1	1
Endocarditis	-	1	1
Leucocytosis (>11,000/µl)	5	3	8
Low platelet count (≤150,000 millions/ml)	6	5	11
Increased liver enzymes (AST/ALP/AP)	4	3	7
RF (n=15)	-	-	-
CRP (n=18)	1	-	1

*P<0.05. AST, aspartate transferase; ALT, alanine transferase; AP, alkaline phosphatase; RF, rheumatoid factor; CRP, C-reactive protein; QF, Q fever

Of the 41 patients who provided paired samples, nine (22.0%) were diagnosed with acute QF and 10 (24.4%) were regarded as past QF (Table I). IgM phase II antibodies to *C. burnetii* remains for several months, unlike other infections, hence the relevance of IgG phase II over IgM phase II has been stressed[7].

In conclusion, the present study showed presence of QF in and around Puducherry. Notwithstanding the sophistication, exorbitant cost and the need for importing the kits, acute QF diagnosis mandates the performance of IFA for screening and real-time PCR for confirmation. Whenever there is a strong clinical suspicion of acute QF, search for *C. burnetii* DNA in the clinical samples by PCR is mandatory, even if the patient's acute samples are negative for IgM and IgG antibodies in IFA. All attempts should be made

to procure convalescent serum samples from patients by properly educating them. The sample size (n=41) in our study was a limitation and inclusion of more number of febrile patients who volunteer to provide paired samples could reveal the true incidence of QF.

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