Original Article

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Microbiology Section

Diagnosis of Acute Q Fever by Detection of *Coxiella burnetii* DNA using Real-Time PCR, Employing a Commercial Genesig Easy Kit

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ABSTRACT

Introduction: Query (Q) fever is an important zoonosis and a cause of concern for humans, due to the potential bioterrorism threat posed by the causative agent, *Coxiella burnetii*. Because of the danger of contracting the illness, isolation attempts are seldom made. Serological and molecular diagnostic tests are the main option.

Aim: To study the prevalence of acute Q fever in Puducherry and surrounding districts of Tamil Nadu, India, employing a new commercial Real-Time Polymerase Chain Reaction (RT-PCR) kit and confirming it by the gold standard Immunofluorescence Assay (IFA).

Materials and Methods: Acute phase blood samples from 72 consecutive febrile patients and 24 healthy individuals were included in this prospective study. DNA was extracted from the buffy coats and preserved at -80°C. Detection of *C. burnetii* was carried out employing a commercial Real-Time PCR kit. Serum samples were tested for IgM (Phase I+II) and IgG (Phase I+II) by QM-120 and QG-120, *Coxiella burnetii* IFA Fuller Laboratories, California, USA. Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were calculated keeping IFA as the reference.

Results: Presumptive diagnosis of acute Q fever was made in two febrile patients by the Genesig Easy kit (2.78%). In addition to these two PCR positive cases, one more patient was positive for both Phase II IgM and Phase II IgG antibodies by the gold standard IFA. All 24 healthy controls were negative for Q fever by both PCR and IFA. The sensitivity, specificity, NPV and PPV for Genesig Easy kit PCR were: 66.67%, 100%, 100% and 98.57 % respectively against IFA as the reference.

Conclusion: The true prevalence of Q fever in India and other developing countries is poorly understood, owing to the difficulties in the diagnosis of this infection. Since molecular diagnostic tests have good specificity and are mandated for confirmation of single acute samples, validation of commercial Q fever PCR kits is the need of the hour. Genesig Easy kit in our hands was found to be reliable with the moderate sensitivity and high specificity. Performing both PCR (with acute specimens) and IFA (with paired sera) would be ideal for Q fever diagnosis.

Keywords: Coxiellosis, Immunofluorescence assay, Quantitative polymerase chain reaction, Zoonosis

INTRODUCTION

The causative agent of Q fever, *Coxiella burnetii*, is a Gram-negative obligate intracellular bacterium highly resistant to the environment. Q fever is reported worldwide with the singular exception of New Zealand [1]. An outbreak of Q fever in Danish goat, leading to killing of large numbers of infected goats [2,3] and reports of coxiellosis in different countries across the globe have raised the awareness level of Q fever throughout the world [4,5]. *C. burnetii* is listed as one of the potential agents of bioterrorism and classified under "Category B pathogen" requiring biosafety Level 3 containments for the isolation/culture [6]. Hence, isolation attempts are limited to very few research/reference laboratories. The serological diagnosis is made, mostly by tests like Enzyme Linked Immunosorbent Assay (ELISA), Complement Fixation Test (CFT) and IFA [4-11]. While IFA is considered as the gold standard, direct proof of *C. burnetii* in the blood by PCR is considered as a confirmatory test especially in single acute blood specimens [12-21]. Domestic livestock like cattle, buffalo, sheep and goat are the primary sources. Human acquires infection through inhalation of aerosols from environment by aborted materials or consumption of unpasteurized milk and milk products. The infection is mostly asymptomatic. Symptomatic patients present as acute or chronic Q fever. Pneumonitis and fever are the common features of acute Q fever and other manifestations includes cough with expectoration, malaise, atypical pneumonia, headache, fatigue and abdominal pain. Endocarditis or hepatitis are the presentation of chronic Q fever [4,6].

Recent recommendation for acute Q fever diagnosis with single serum samples mandates application of PCR [14,19]. There are only a few reports from India regarding QF diagnosis on the basis of molecular detection of *C. burnetii* DNA in human/animals/animal feed [15-18,22-29]. The objective of the present study is to determine the prevalence of Q fever and validate the usefulness or otherwise of a new commercial Real-Time PCR kit (Genesig Easy kit) for the diagnosis of acute Q fever in a tertiary care teaching hospital catering to patients from parts of Puducherry and surrounding districts of Tamil Nadu, India.

MATERIALS AND METHODS

This investigation was initiated to search for *C. burnetii* DNA in patients with acute febrile illness of one to two weeks duration. This investigation was conducted in the Department of Microbiology and Paediatrics, Mahatma Gandhi Medical College and Research Institute (MGMC and RI), Puducherry, a tertiary care teaching hospital and Department of General Medicine, Indira Gandhi Govt. General Hospital and Post Graduate Institute (IGGGH and PGI), Puducherry, India. The work was carried out between March, 2016 to March, 2017. After getting approval from the Institutional Human Ethical committee (IHEC) the research was initiated. Informed written consent was obtained from patients with complaints of fever. The patients came from two health care institutions: a private sector medical college hospital, MGMC and RI and Indira Gandhi Govt. General Hospital and Post Graduate Institute (IGGGH and

PGI), Puducherry and belong to various places in Puducherry and neighbouring districts of Tamil Nadu, India, who provided only acute blood samples. Specimens from consecutive patients were collected during the period of March 2016-December 2016 and molecular work was carried out upto March, 2017. A total of 72 febrile patients and 24 healthy individuals were included in this study. These healthy participants were voluntary blood donors (17 male and 7 female) in the age group of 20-45 years. Information regarding contact with domestic livestock/pets was solicited from the participants at the time of recruitment.

Inclusion criteria: Cases with high-grade fever with or without chills and rigor; fever with pneumonia/pneumonitis; fever with rash/hepatosplenomegaly/jaundice/lymphadenopathy/thrombocytopenia; fever with constitutional symptoms like malaise, myalgia, nausea and vomiting. Other clinical parameters that were determined include: headache, cough and expectoration, abdominal pain and complications.

Exclusion criteria: Fever due to Urinary tract infection/ Malaria/ Enteric fever; culture positive bacterial pneumonia; patients with other blood stream infections; bleeding disorders and fever of more than four weeks duration (pulmonary tuberculosis). Some common causes of Fever of unknown Origin (FUO) were excluded by performing appropriate tests like Serology for enteric fever, dengue and leptospirosis, blood culture and urine cultures, blood examination for malarial parasite (Smear and antigen detection by immunochromatography) etc.

Quantitative polymerase chain reaction (qPCR): About four ml of blood was collected in EDTA tubes, centrifuged at 2500 rpm for five minutes and the buffy coat layer was transferred to a sterile tube and kept frozen at -20ºC until further use.

DNA extraction: About 200 µl of buffy coat samples were used for genomic DNA extraction using QIAamp DNA Blood Mini Kit (Qiagen, Germany) as per the manufacturer's protocol. The purity of the extracted DNA was determined by calculating absorbance (A) A260/A280 ratio, which was in the range of 1.7-1.8 for all the samples. These samples were aliquoted and stored at -80°C. We purchased *Coxiella burnetii* q16 Genesig easy kits (Primer design Ltd., Southampton, United Kingdom). DNA extracts of patients and controls were taken to Diagnostic Services, 3i Molecular Solutions, Bengaluru for performing real-time PCR using a portable Genesig q16™ qPCR machine. Positive and negative controls were included in the kit. Additionally, in each run *C. burnetii* DNA (Nine Mile Phase I RSA 493) obtained from Bioscience, Bratislava, Slovakia was included.

A reaction mix of 20 µl was prepared by adding 10 µl of 2 x osaig™ Master mix (which contains *C. burnetii* specific primers/probe) and 10 µl of template DNA (inclusive of Internal extraction control DNA). PCR reaction was carried out with an initial denaturation of 95ºC for two minutes followed by 45 cycles of denaturation 95ºC for 10 seconds and combined annealing and extension of 60ºC for one minute as described by manufacturer instructions. The technique of combining annealing and extension as a single step has been reported by Cherupanakkal C et al., [30]. Primer design's oasig™ Master Mix contains a Taq enzyme with a high level of polymerase activity at 60°C. This allows the extension step to proceed at this temperature. Furthermore, the exonuclease activity that hydrolyses the probe, producing the amplification curve, was most active at 60°C. Along with the primary assay design criteria, this ensures that the *C. burnetti* kit will exhibit optimal performance levels, when the annealing and extension steps are combined. It targets DNA gyrase subunit A which encodes 97 kDa gyrA gene (Accession no: GU324985, Context length–138 bp and Anchor nucleotide – 161) (obtained from the Primer design Ltd., technical team).

IFA: About 2 ml-3 ml blood was collected in sterile tubes without anticoagulant. After clotting, serum was separated and stored at -20°C. QM-120 and QG-120, *Coxiella burnetii* IFA kit (Fuller Laboratories, California, USA) were used. IFA IgM (Phase I+II) and IgG (Phase I+II) was performed strictly adhering to the kits' technical brochures.

STATISTICAL ANALYSIS

Sensitivity, Specificity, PPV and NPV of this kit, was determined keeping IFA as the gold standard (Vaidya VM et al., [15]). Patients' clinical details and laboratory test results were analysed by Fisher-exact test, using Graph Pad Quick Calcs (GraphPad Software Inc, USA) and p-values of ≤0.05 were considered as statistically significant.

RESULTS

A total of 96 samples comprising of 72 acute samples from febrile patients and 24 healthy controls were tested for *C. burnetii* DNA by qPCR. Diagnosis of acute Q fever was made in two adults whose blood samples were positive for *C. burnetii* DNA with the Genesig Easy kit (2.78%). Cycle threshold (Ct) values of the positive samples were 35.0 and 36.2 respectively. One adult male patient had Lower Respiratory Tract Infection (LRTI) and fever of four days duration with chills and rigor, headache, cough, expectoration and pneumonitis. He had leucopenia (3,700/mm^3) and thrombocytopenia with a platelet count of 52,000/mm^3. The second patient (female) gave a history of domestic animals contact with cow and sheep. She had fever of eight days duration with chills and rigor, myalgia, headache, abdominal pain, nausea and low platelet count (16,000/mm^3). The above two patients were positive for both IgM (Phase II) and IgG (Phase II). A third case of acute Q fever was made purely on serological evidence of IgM and IgG positivity (Phase II). This male patient had fever of 14 days duration and presented with respiratory symptoms.

[Table/Fig-1] analyses difference between children and adults among 72 febrile patients with reference to their clinical signs

Clinical symptoms	Children ≤ 18 years (n= 9)	Adults ≥ 19 years (n= 63)	Total n=72 (%)	p-value
Fever ≤ 14 days	9	50	59 (81.4)	0.197265
Chills and rigor	6	45	51 (70.8)	0.714347
Myalgia	2	11	13 (18.0)	0.661308
Headache	2	25	27 (37.5)	0.468164
Cough and Expectoration	3	25	28 (38.8)	0.7147
Pneumonitis	0	14	14 (19.4)	0.190141
Abdominal Pain	3	31	34 (26.3)	0.485184
Hepatomegaly	1	7	8 (11.1)	1
Splenomegaly	0	2	2 (2.7)	0.5877
Vomiting	4	22	26 (36.1)	0.713866
Rash	1	2	3 (4.1)	0.334155
Nausea	1	14	15 (20.8)	0.673839
Lymphadenopathy	2	0	2 (2.7)	0.014085*
Leucocytosis (>11,000 cmm)	2	17	19 (26.3)	0.7617
Low Platelet count (<1.5 lakhs/mm^3)	3	18	21 (29.1)	0.714347
Increased Liver Enzymes [£](AST/ ALT/AP)	5	17	22 (30.5)	0.120522
Creatinine (>1.0 mg/dL)	0	2	2 (2.7)	0.5877
Complications	0	2	2 (2.7)	0.5877

[Table/Fig-1]: Clinical and laboratory details of febrile patients (n=72).
*p-values <0.05 were considered significant.
[£]Aspartate Transferase (AST)/Alanine Transferase (ALT)/Alkaline Phosphatase (AP)

and symptoms as well as laboratory findings. The only statistical significant finding was more number of children had lymphadenitis than adults (p=0.014085). Mean age (SD) of participants was 34.25±16.31 and male-female ratio was 7:11. Sensitivity, specificity, PPV and NPV were 66.67%, 100%, 100% and 98.57% respectively keeping IFA results as the gold standard. We find this commercial kit reliable with a moderate sensitivity and 100% specificity.

DISCUSSION

Q fever is not a notifiable disease in several countries including India. Two reviews of Q fever in man and animals of India appeared in 1978 and 1980, giving detailed account of seroprevalence as well as tests employed by earlier workers. The late seventies and early eighties witnessed several reports of Q fever in human/animals from several states like Punjab, Haryana, Kerala, Karnataka, Uttar Pradesh, Maharashtra, Delhi, Orissa [31-39] and Rajasthan [40]. Evidence of animal and human abortions due to *C. burnetii* based on PCR and Immunofluorescence tests [15,22,41] and Q fever endocarditis are recorded in Indian literature [42]. A recent report from Chennai of neonatal sepsis caused by Q fever is an evidence of this zoonosis in Southern India [23]. For detection of *C. burnetii* DNA in clinical samples, PCR assay should be performed within 14 days of febrile illness, which is prior to the appearance of antibodies. PCR is a highly valuable tool for early diagnosis of Q fever compared to Immunofluorescent Assay IFA the 'gold standard' for serodiagnosis of acute and chronic Q fever [14,15,21,43]. In the course of our Task Force ICMR project on rickettsial diseases, we experienced unreliability of *C. burnetii* Phase II IgM ELISA kits which were available in India. Our preliminary study using the gold standard IFA (IgM Phase II) revealed the seropositivity of 15.45% among 58 febrile patients [44]. According to the recent algorithm, seroconversion for Q fever is defined as an initial sample being IgG phase II negative but positive in the follow up sample [14,19-21,43]. According to Raven CF et al., PPV of single acute and isolated IgM phase II result by IFA or ELISA was 65% and 51%, respectively and hence, do not adequately predict acute Q fever and therefore cannot be used as a diagnostic criterion [43]. Our patients' compliance in making a second visit to provide convalescent blood sample is very poor. For single acute samples, detection of DNA in the blood by PCR rather than antibody detection by IFA is more apt especially if the duration of febrile illness is ≤14 days [14,19,24]. Hence, this study has concentrated on molecular diagnosis of acute Q fever with single serum samples. Vaidya VM et al., reported that the performance of molecular detection based on IS1111 gene in Light Cycler PCR was 25.68% sensitivity and 100% specificity [15]. Genesig Easy kit has shown 66.67% sensitivity which could be considered to be moderate, and a high specificity of 100%. The q16 Genesig portable Real-Time PCR machine can be easily operated from PC, Mac via network or stand alone with a USB drive. However, kits other than Genesig easy kit are not suitable, since according to the technical brochure of the kit, "The genesig easy kit range is the simplest to use version and is designed specifically for use on the genesig q16 instrument" [45]. Edouard S et al., suggested that sensitivity of qPCR could be increased from 36% to 44% in early seronegative patients by preserving the sera by lyophilization instead of other methods [20]. We could not lyophilize our serum samples but only preserved them at -20°C for a period ranging from three to nine months. Therefore, DNA degradation of our sera is a possibility and PCR could have missed a few cases. Specificity of Genesig™ easy kit as per the claim of the kit's manufacturers is 100% and this kit has picked up low copy numbers of *C. burnetii* DNA (5 and 8 copies) in our study. DNA Gyrase Subunit A, which is targeted by GenesigTM kit is an enzyme which is well known in Gram negative bacteria and it contains 97 kDa gyrA gene which is present in the Cub Q212, a reference strain [25]. Instances are there in world literature wherein PCR/IFA positivity in acute Q fever presents the following possibilities in febrile illness of ≤14 days duration: PCR only positive/IFA only positive/Both PCR and IFA positive (Jager MM et al., Schneeberger PM et al., and Alves J et al., [14,19,24]). Genesig easy kit has shown more than moderate level of sensitivity 66.67% and 100% specificity. Because of the portable nature of this small sized instrument, Q fever PCR work is feasible in ordinary laboratories. Ideally, Q fever PCR is the preferred choice for febrile illness ≤14 days and IFA for paired sera with interval of three to four weeks. In our experience, this kit is reliable with a satisfactory performance.

LIMITATION

We could not perform the follow up of two QF positive patients due to time lapse of seven months from sample collection to PCR work. Since, convalescent serum was not collected from any of the 72 patients, QF serology with paired serum samples was not carried out. Seroconversion for Phase II IgG in convalescent samples (which could not be collected) might have increased the seroprevalence percentage of acute Q fever. This is due to seroconversion of Phase II IgG antibodies in the convalescent sample. This problem is faced by several researchers due to failure of the patients to voluntarily come forward to provide convalescent sample. Most of our patients have to travel between 20 Kilometers to 150 kilometers to reach our hospital and return back home.

CONCLUSION

The present study has revealed the low prevalence of acute Q fever in patients attending our hospital. Positive IFA serology in three patients and *C. burnetii* DNA detection among two of them confirm the present status of Q fever in this part of Southern India. Further study with large number of patients, who volunteer to provide both acute as well as convalescent blood samples, might perhaps through light on the true prevalence of this zoonosis. This kit, with its moderate sensitivity and high specificity could be very useful in laboratories, which lack facilities for in-house PCR work. Presently QF qPCR kits need to be imported and are expensive. Easy availability of these kits at affordable cost would encourage more workers to take up Q fever research.

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