First molecular evidence of *Coxiella burnetii* in patients with atypical pneumonia, India

*Coxiella burnetii* is the causal agent of Q fever, a worldwide zoonosis and considered a public health problem in many countries. Domestic animals and pets are the most frequent source of *Coxiella* infections in humans (Maurin & Raoult, 1999). Transmission to humans is usually via inhalation of contaminated aerosols from urine, faeces, milk or birth products, or less commonly by ingestion of unpasteurized milk from infected farm animals. Q fever is characterized by acute and chronic courses. Acute Q fever is often asymptomatic or manifests as an influenza-like unrecognized illness, hepatitis or atypical pneumonia (Maurin & Raoult, 1999). Blood culture-negative endocarditis, vascular infections, osteoarticular involvement and chronic liver diseases are the main clinical presentations of chronic Q fever (Maurin & Raoult, 1999). During the largest outbreak of Q fever in the Netherlands, pneumonia (61.5%) was the most common presentation (Lai et al., 2013). Although *C. burnetii* has been implicated in atypical pneumonia reported worldwide (To et al., 1996; Lee et al., 2002; Lai et al., 2013; Chen et al., 2015), its distribution in India is not defined.

In India, the first confirmed human case of Q fever was reported in 1954 (Kalra, 1954). Subsequently, there have been only a few reports of human Q fever in the country (Stephen et al., 1980; Vaidya et al., 2008). The serological screenings in most of these reports are largely based on conventional tests such as a capillary agglutination test and complement fixation test. A complement fixation test may fail to detect primary Q fever during the early phase of illness as well as in some chronically infected patients, and therefore requires acute- and convalescent-phase serum samples for reliable interpretation of results (Péter et al., 1985; Fournier et al., 1998). However, in India, paired serum samples to detect a rise in titre are almost never sent to the laboratory for diagnostic confirmation. It is therefore logical to assume that infection by *C. burnetii* has remained grossly underdiagnosed in the Indian population. To the best of our knowledge, there is no report from India of *C. burnetii* being responsible for atypical pneumonia; this could possibly be attributed to the fact that atypical organisms have rarely been isolated from patients with community-acquired pneumonia, confounded by the lack of facilities for culture/serology of these organisms. We therefore investigated the aetiological role of *C. burnetii* in patients with atypical pneumonia by molecular methods, such as TaqMan real-time PCR and conventional PCR.

Blood samples from patients presenting with atypical pneumonia at M. S. Ramaiah Medical College and Hospital, Bangalore, Karnataka, India, between January 2014 and April 2015 were collected aseptically by venous puncture after obtaining permission from the Institutional Ethical Committee (no. MSRM/EC/2014). The samples were centrifuged at 2000 g for 10 min at 4 °C. Serum was separated and stored at −20 °C until assayed. All samples were screened using a Pneumoslide IgM test (Vircell), an indirect immunofluorescence technique for simultaneous IgM detection of 9 pathogens of the respiratory tract comprising *Legionella pneumophila* serogroup 1, *Mycoplasma pneumoniae*, *C. burnetii*, *Chlamydia pneumoniae*, adenovirus, respiratory syncytial virus, influenza A virus, influenza B virus and parainfluenzaviruses 1, 2 and 3. The Pneumoslide IgM test is a reasonably sensitive and highly specific technique for detection of viral or atypical bacterial pathogens (El-Sahriy et al., 2006).

The gold standard for serological diagnosis of an infectious disease is either seroconversion or a fourfold rise in antibody titre. The reference test for serological diagnosis of Q fever is the immunofluorescence assay (Maurin & Raoult, 1999). Antibodies are expressed against phase II antigens during the acute infection and against phase I antigens in the established infection. However, an important drawback to serological diagnosis of acute Q fever is the lag phase in antibody response of 7–15 days after onset of clinical symptoms (Maurin & Raoult, 1999); in addition, solitary IgM phase II serology results are not sufficient for diagnosis and notification of a confirmed case of Q fever (Raven et al., 2012). Moreover, serological cross-reactivity with other intracellular pathogens, including *Bartonella* spp., *Legionella mic-dadei*, *Rickettsia spp.*, *Chlamydia pneumoniae*, cytomegalovirus and Epstein–Barr virus, has been reported in Q fever patients (Lai et al., 2013). In the present study, 77 serum samples from atypical pneumonia cases that were negative by Pneumoslide IgM test were subjected to TaqMan real-time PCR based on amplification of a 70 bp fragment of repetitive sequence IS1111 of *C. burnetii* as described by Schneeberger et al. (2010). PCR was carried out in a StepOne real-time PCR system (Applied Biosystems) using TaqMan Universal Master mix II (Invitrogen) according to the manufacturer’s instructions. The results were analysed with StepOne software v.2.3 (Applied Biosystems). Of 77 samples tested, two (S9 and S22; 2.6%) were found to be positive with cycle threshold (C) values of 29 and 25, respectively. These samples were further confirmed by conventional PCR targeting repetitive element IS1111 of *C. burnetii* (Lorenz et al., 1998), which was used to amplify a 243 bp fragment of the IS1111 element. Double-pass sequencing of PCR products was carried out using a 3730 DNA Analyser (Applied Biosystems). The sequence of amplicons showed 100% nucleotide identity with the reference strain of *C. burnetii* (GenBank accession nos LN999998 for S9 and LN999999 for S22). These two samples were also found to be positive by SYBR Green real-time PCR targeting another multi-copy gene target,
JS30A spacer, with C values of 30 (S9) and 27 (S22). PCR on serum has been shown to be positive mostly in patients that have not yet mounted an antibody response (Schneeberger et al., 2010). It was difficult for us to further confirm these cases using an alternative serological test, especially in the absence of paired sera.

When the epidemiological history of the positive patients was investigated, it was observed that patient S9 was a 64-year-old male, a rancher by occupation, and presented with a 5-day history of fever, dry cough, shortness of breath and hepatosplenomegaly; echocardiography revealed features suggestive of endocarditis. Patient S22 was a 72-year-old farmer suffering from fever and breathlessness for 6 days with hepatosplenomegaly; a chest X-ray showed bilateral lower zone consolidation. The findings of the present study suggest that patients with atypical pneumonia should be screened by molecular methods in the absence of facilities for culture and serology with paired sera.

Our preliminary study using a small number of samples suggests that C. burnetii is the prevalent causative organism of atypical pneumonia in India. We recommend that C. burnetii PCR with serum samples should be routinely included in the diagnostic work-up of a patient with suspected atypical pneumonia. Further studies, using more samples from diverse geographical locations and employing molecular methods specific for C. burnetii, are necessary to elucidate the epidemiology of Q fever in India.

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