Coxielliosis in domestic livestock of Puducherry and Tamil Nadu: Detection of Coxiella burnetii DNA by polymerase chain reaction in slaughtered ruminants

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Background and Aim: In the course of our Indian Council of Medical Research project on coxiellosis in Puducherry and Tamil Nadu, 5.64% goat, 1.85% sheep, 1.06% buffaloes, and 0.97% cattle were positive for Coxiella burnetii DNA by polymerase chain reaction (PCR), with a band at 243 bp. Only one buffalo serum sample was positive for C. burnetii DNA in those antibody positive specimens employing an imported commercial C. burnetii PCR kit (Genekam Biotechnology AG, Duisburg, Germany). An in-house Trans-PCR was included in the study for comparison.

Results: A total of 15 antibody positive and three antibody-negative serum samples belonging to 11 goat, 4 sheep, 1 cattle, and 2 buffaloes were tested in duplicate for the presence of C. burnetii DNA by the commercial agar gel PCR kit (Geneekam) and an in-house Trans-PCR. Only one buffalo serum sample was positive for C. burnetii with a band at 243 bp in in-house Trans-PCR.

Discussion: Seropositivity for C. burnetii need not necessarily translate into infectivity status of the animal. Conversely, seronegative ruminants can shed C. burnetii. Rapid disintegration of C. burnetii DNA during the storage period is an important impediment in QF-PCR research. This is the first time the performance of this commercial PCR kit is being validated in India.

Conclusion: Commercial PCR kit, Genekam did not identify any positive sample, probably because it targeted a larger amplicon of 687 bp.

Keywords: Coxiella burnetii DNA, coxiellosis, Trans-polymerase chain reaction.

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surveys by Kalra and Taneja [17]. 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 [18,19]. The late seventies and early eighties witnessed several reports of Q fever in human/animals from several states such as Punjab, Haryana, Rajasthan, Kerala, Karnataka, Uttar Pradesh, Maharashtra, Delhi, Orissa, and the latest from Rajasthan (2003), Tamil Nadu (2008), and Puducherry (2014) [20-32]. Evidence of animal and human abortions, neonatal septicemia, endocarditis, and atypical pneumonia due to C. burnetii based on immunofluorescence test/polymerase chain reaction (PCR) are recorded in recent Indian literature [4,5,33,34].

In the recent times, coxiellosis in animals have been reported from several countries such as Bangladesh, Iran, Brazil, Turkey, USA, Greece, Bulgaria, Switzerland, Italy, and The Netherlands [14,35,36]. An outbreak of Q fever in Danish goat, leading to killing of 51,680 infected goats and reports of coxiellosis in different countries across the globe have raised the awareness level of Q fever throughout the world [36]. Nearly, 583 abortions had occurred due to C. burnetii infection in small ruminants between 2002 and 2011. An observation of C. burnetii infection in Swiss animals by screening of milk samples shows that mostly it occurred in <5% of cattle products and absent in sheep or goat samples [37].

Seroprevalence studies of coxiellosis in several countries including India were based on specific and sensitive serological tests such as capillary agglutination test, complement fixation test, enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence assay, molecular tests like PCR, real-time PCR, and loop-mediated isothermal amplification [4-43]. Our aim of this preliminary communication is to examine a small number of seropositive ruminants for C. burnetii DNA. Evaluation of a commercial and imported conventional PCR kit is done for the first time in India and compared with an in-house prepared Trans-PCR.

Materials and Methods

Ethical approval

Institute’s Animal Ethical Committee had given approval for this work.

Study area

This study was conducted in the Microbiology Department of a tertiary care super specialty teaching hospital at Puducherry during January 2014 to December 2015.

Collection of blood samples

Blood samples were collected from domestic livestock at the time of slaughtering from various private/government/municipal abattoirs as well as mutton shops located in different areas of Tamil Nadu and Puducherry. A total of 772 blood samples were collected comprising 216 sheep, 195 goat, 206 cattle, and 188 buffaloes.

Test procedure for ELISA and PCR

ELISA was performed with Q fever antibody ELISA Test Kit, (IDEXX, Liebefeld, Switzerland). The ELISA wells were coated with C. burnetii Phase I and II antigens and it was carried out as per the procedure outlined by the kit manufacturer and as per earlier report [6].

DNA extraction

About 200 µl serum samples were used for the genomic DNA extractions as per the manufacturer’s protocol. Carrier DNA was added to the serum sample before extraction to maximize DNA yield, as recommended by the 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. The samples were aliquoted and stored at −80°C till further use.

Kit-based Trans-PCR

Trans-PCR for the samples was done in duplicates using C. burnetii PCR kit (GeneKam, Disburg, Germany) as per manufacturer’s protocol. The reaction conditions for the PCR were as follows: Denaturation at 20 s at 95°C, annealing for 60 s at 50°C, and extension for 120 s at 72°C for 30 cycles. The PCR was carried out using C1000 Thermocycler, Bio-Rad, USA, and the PCR product was visualized on agarose gel by ethidium bromide staining.

In-house Trans-PCR

In-house PCR was carried out using primers (Sigma-Aldrich, Bengaluru) for amplifying IS1111 transposon–like repetitive element of C. burnetii [2,9,10]. The sequence of the primers used was Trans 1: 5’-TATGTATCCACCGTAGCCAGTC-3’ and Trans 2: 5’-CCCAACAACACCTCCTTATTC-3’; Trans 3: 5’-GTAACCGATGGCAOGCGAT-3’ and Trans 4: 5’-CCACCGCTTTCGCTGCTA-3’. The PCR reaction was carried out in 25 µl of reaction mixture with 12.5 µl ×2 Taq DNA polymerase PCR kit (ampliqon), 1 µl of forward primer and reverse primer each, 5 µl template DNA, and 5.5 µl molecular grade distilled water. The PCR conditions were as follows: Denaturation at 95°C for 30 s, annealing at 65°C for 40 s extension at 72°C for 30 s for 35 cycles, and a final elongation step at 72°C for 5 min. The PCR was carried out using C1000 Thermocycler, Bio-Rad, USA and the PCR product was visualized on agarose gel by ethidium bromide staining.

Results and Discussion

In this study, we used four primers Trans 1, 2, 3, and 4 which target IS1111 transposon-gene repetitive element for detection of C. burnetii in serum samples. 18 samples, 15 antibody positive, and three
antibody-negative serum samples belonging to 11 goat, 4 sheep, 1 cattle, and 2 buffaloes were tested with ready to use conventional Genekam PCR kit with the primer set of Trans 1 and 2, with an expected amplicon size of 687 bp, but none of them were positive. The positive control provided in the kit, as well as C. burnetii DNA (Bioscience, Bratislava) gave clear band at 687 bp (Figure-1). Negative controls were added in each run to avoid DNA contamination. When our in-house Trans-PCR was performed with primer set of Trans 1 and 2, all samples were negative except the positive controls. However, when the experiment was repeated with the second set of primers, viz., Trans 3 and 4, one seropositive buffalo had C. burnetii DNA (Figure-2).

In this study, we evaluated commercially available, ready to use C. burnetii conventional PCR for the first time in India and found it unsatisfactory for Indian ruminants, which is most likely because it targets a larger molecular weight amplicon size 687 bp. Q fever is on the increase and included in the list of notifiable diseases in some European countries [36,39]. Largest outbreak in Bulgaria and Europe lead to the investigators to find out a high seroprevalence of C. burnetii antibodies in humans as well as animals [36,42]. According to Bellini et al., only 14% C. burnetii antibody positive (by ELISA) ruminants had C. burnetii DNA in the real-time PCR assay [44]. However, Kargar et al. reported that the primers Trans 1 and 2 are highly sensitive and showed 17.14% positivity when compared with Com1 and Coc-PCR with only 10% positivity for milk products [9]. However, Kilic et al. reported that Trans 1 and 2 PCR could detect only 2% C. burnetii DNA in the organs of infected animals and observed that IS1111 gene was circulating among the domestic sheep and goat [40]. Gunaydin et al. reported that Trans PCR 687 bp displayed a negative result in animal serum samples which are seropositive for C. burnetii antibodies [45]. According to a recent report by Chakarabarthy et al., the overall seroprevalence of coxiellosis in Bangladesh was 7.6% and 6.1% for goats and cattle, respectively. However, none of seropositive samples were positive for C. burnetii DNA in real-time PCR [35]. Two researchers observed that C. burnetii appears to be quite frequent in blood as well as milk products of cattle [14,35]. Kim et al. accounted year wise prevalence of coxiellosis in milk products of cattle as 52.8%, 23.5%, and 31.3% in 2002, 2003, and 2004, respectively [14].

There is not much of a progress in India in the molecular diagnosis of coxiellosis/Q fever, except for few reports from Uttar Pradesh, Karnataka, and Tamil Nadu [4-7,31,34]. By the application of molecular diagnosis using Trans-PCR, Indian researchers reported prevalence of Q fever 21.6% of human abortions, 11.05% of domestic animals with reproductive disorders, and 2.8% of patients with atypical pneumonia [4,5,34]. Our study points to the fact that Trans 3 and 4 are sensitive than Trans 1 and 2, as it is a short fragment in the most conserved region of C. burnetii DNA. Use of 243 bp or even smaller 70 bp amplicon [34] could perhaps pick up more positive cases in humans as well as domestic livestock. Das et al. isolated C. burnetii from the aborted fetuses of 4.54% cattle and 8.33% buffaloes [6]. IS1111 Transposon-repetitive element is best known target for detection of C. burnetii DNA in patients with active infections [6,40]. Degradation of preserved C. burnetii DNA is a major drawback in the molecular diagnosis of coxiellosis. It is known that while C. burnetii antibody positive animals need not necessarily shed this organism, antibody negative livestock can shed this bacterium in their secretions/excretions [32]. In our preliminary work, only a small number of 18 ruminants’ serum samples were used for detection of C. burnetii DNA. The study could be expanded to cover more number of animals so as to get a better and clear picture of the prevalence of this zoonosis.

Conclusion
This preliminary communication records a low (6.67%) C. burnetii DNA among antibody positive ruminants. The use of smaller amplicon size of 243 bp and perhaps even much smaller ones might result in a higher percentage of positivity. The large sized amplicon of 687 bp is perhaps a reason for the failure of the commercial kit to detect any positive case.
Authors’ Contributions
SS, JP, PXA planned, designed, and conducted data interpretation. BS and JP performed sample collection. BS, JP, AA carried out sample analysis. PXA and PP edited the manuscript. All authors read and approved the final manuscript.

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Competing Interests
The authors declare that they have no competing interests.

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