Research Article

Screening of Foods of Animal Origin for Coxiella burnetii in India

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INTRODUCTION

Q fever (query fever), erstwhile classified as a rickettsial zoonosis, is now better known as an OIE notifiable bacterial zoonotic disease caused by the most contagious, Gram–negative, obligate, intracellular bacterium – Coxiella burnetii (OIE, 2010; ILRI, 2012). The disease has a worldwide negative, obligate, intracellular bacterium – Coxiella burnetii (OIE, 2010; ILRI, 2012). The disease has a worldwide incidence with the notable exception of New Zealand (Hifi link et al., 1993; Norlander, 2000; Angelakis and Raoult, 2010). It remains endemic in many parts of the world and its prevalence has been confirmed in at least 51 countries including India (Acha and Szyfres, 2003; Marrie, 2003). Of late, Q fever is emerging or re-emerging in several countries worldwide (Arri cu–Bouvery and Rodolakis, 2005; Gwida et al., 2012; Natale et al., 2012). It is noteworthy that the prevalence of Q fever has increased many–fold in recent times not only in the human beings and animals(Gwida et al., 2012), but also in foods of animal origin(Kim et al., 2003, Rahimi and Doosti, 2012) in humans, Q fever manifestations range from asymptomatic or mild flu-like illness to chronic fatal endocarditis (Angelakis and Raoult, 2010).

Major reservoirs of C. burnetii include farm animals and pets (Agerholm, 2013), and the infection gets transmitted to humans mainly through inhalation of contaminated aerosols of infected parturition materials or dusts containing C. burnetii shed by infected animals (Tissot–Dupont et al., 1999; Angelakis and Raoult, 2010; Agerholm, 2013). Moreover, continual shedding of the bacteria in the milk of infected animals (Maurin and Raoult, 1999; Kim et al., 2005), predominantly of cattle, for prolonged time periods ranges from 1 month (Arri cu–Bouvery and Rodolakis, 2005) to 3 months (Angelakis and Raoult, 2010).

The pathogen has also been detected in foods of animal origin like milk (Ongor et al., 2004; Kim et al., 2005; Fretz et al., 2007), meat (Schaal, 1977), eggs and their products (Tatsumi et al., 2006; Rahimi and Doosti, 2012). The prevalence of pathogen in milk has been reported to range from a high of 94% in bovine milk bulk tank samples in USA (Kim et al., 2003) to 6.2% in bovine and 1.8% caprine bulk milk samples in Iran (Rahimi et al., 2009), 4.7% of bovine milk samples in Switzerland (Fretz et al., 2007), and 3.5% of ovine milk samples in Turkey (Ongor et al., 2004). The screening of eggs and their products for C. burnetii by PCR revealed positivity in 4.2% egg samples and 17.6% of the mayonnaise specimens in Japan (Tatsumi et al., 2006) and 1.5% of hen eggs and 7.7% duck eggs in Iran (Rahimi and Doosti, 2012).

Molecular method like PCR has become a useful tool with wide applicability in direct detection of C. burnetii DNA in different samples for diagnosis of Q fever because of its high specificity and sensitivity (Levy et al., 1991), as it can detect specific DNA sequences that may be present even in very low numbers in the fresh or frozen samples including milk, vaginal swabs, feces (Beri et al., 2000, 2001). Of late, a Trans–PCR assay, targeting the transposon– based IS1111 insertion sequence of C. burnetii has been widely used for detection of the pathogen...
in various clinical samples (Willems et al., 1994; Berri et al., 2000; Rolain and Raoult, 2005; Vaidya et al., 2008, 2010; Das, 2010).

In India, the association of C. burnetii with clinical conditions in animals and humans has been reported based on the recent and reliable molecular and serological tools (Vaidya et al., 2008, 2010; Das, 2010; Das et al., 2013), however, studies on the prevalence of this important food borne pathogen in different foods of animal origin by sensitive and reliable diagnostic methods are largely lacking. In this context, the present study was envisaged to analyze the occurrence of C. burnetii in different foods of animal origin by PCR, a highly sensitive and specific molecular test for the pathogen detection in milk (Kim et al., 2003; Fretz et al., 2007) and eggs (Tatsumi et al., 2006; Rahimi and Doosti, 2012).

MATERIALS AND METHODS

Collection of sample
In the present study, a total of 591 food samples comprising of milk (n = 318), meat (n = 60) and eggs (n = 13) were collected. Milk samples were randomly collected from 105 cows, 309 buffaloes, 60 ewes and 34 camels belonging to organized or unorganized farms in Goa, Maharashtra, Odisha, Rajasthan, and Uttar Pradesh states of India. Meat samples from female goats (n = 40) with reproductive disorders and indigenous hens (n = 20) were collected from slaughter house and retail market of Bareilly, Uttar Pradesh, respectively. Some eggs (n = 13) of backyard poultry were collected from retail markets of Bareilly. The samples were collected aseptically in screw-capped sterile vials (15 mL) with 10 mL transport medium (sterile Bovarnick’s buffer) or straightly in sterile vials or polybags; and then transported under refrigerated conditions to the laboratory and stored at −20°C until used for PCR testing.

Standard C. burnetii DNA procurement
The DNA of standard C. burnetii Nine Mile strain was obtained from Dr. Eric Ghigo, URMITE-IRD, Faculté de Medecine, France.

DNA Extraction from food samples
Samples of different foods of animal origin were processed for extraction of C. burnetii DNA by using DNeasy Blood and Tissue kit 50 (Qiagen, USA) as per the basic protocol given by the manufacturer.

(i) Milk
The sample of milk (1 mL) was centrifuged at 13,000 x g for 60 min at room temperature, and the layers of cream and milk were removed as recommended by Berri et al. (2000). Buffer ATL (180 mL) was added to the pellet, reaction mixed with proteinase K and then processed for DNA extraction employing DNeasy Blood and Tissue kit 50 (Qiagen, USA).

(ii) Meat
The meat samples (25 mg) were cut into small pieces, put in a 1.5 mL micro centrifuge tube, added with 180 mL Buffer ATL, and processed for DNA extraction.

(iii) Eggs
Firstly, the C. burnetii was extracted from egg samples as per the method described by Fretz et al. (2007) and the resultant pellet was further processed for extraction of DNA as described with milk samples following the protocols of DNeasy Blood and Tissue kit 50 (Qiagen, USA).

Trans-PCR assay
The reported primers, targeting the transposon-like repetitive element of C. burnetii, namely trans-3 (5'-GTA ACG ATG CGC AGG CGAT-3') and trans-4 (5'-CCA CCG CTT CGC TCG CTA-3'), amplifying a 243 bp gene fragment (Lorenz et al., 1998) were synthesized by Sigma Genosys, USA and used in our study. The trans-PCR method of Willems et al. (1994) was employed with some modifications for C. burnetii detection in samples. Briefly, the PCR cycling reactions included an initial denaturation (95°C for 2 min), followed by 35 cycles, each consisting of 94°C for 30s, 61°C for 30s and 72°C for 1 min; and a final extension reaction of 72°C for 10 min. The PCR reaction mixture (25 μL) comprised of 10X PCR buffer (2.5 μL), 10 mM dNTP mix (2.5 μL), 25 mM MgCl₂ (3.0 μL), 10 μM of primers (1.0 μL each), Taq DNA polymerase (0.5 μL ), DNA extract (5 μL) and sterilized nuclease free water for making the full reaction volume. For positive control, the standard DNA of C. burnetii RSA 493 Nine Mile 1 strain was used. The PCR amplification was carried out in a Master Cycler Gradient Thermocycler (Eppendorf, Germany), and the amplified products were subjected to agarose gel (1.5%) electrophoresis with ethidium bromide staining, and visualized under a UV transilluminator (UVP Gel Seq Software, UK).

RESULTS

Trans-PCR assay
The trans-PCR assay, using trans-3 and trans-4 primers of C. burnetii, specifically generated a 243 bp amplified products (Figure 1). Sensitivity testing of the trans-PCR assay using standard DNA of C. burnetii Nine Mile 1 strain of known concentration (70 ng/μL) as well as DNA extracted from the spiked samples, diluted 10-fold, revealed C. burnetii DNA detection in dilutions up to 7 x 10⁻³ ng/μL.

![Figure 1: Trans PCR amplifying 243bp products](image)

Lane 1: Positive standard
Lane 2: Negative standard
Lane 3-5: Positive samples

Of the 591 food samples screened, the trans-PCR assay revealed 24 (4.06%) samples to be positive for C. burnetii, and all of them were milk samples (Table 1). Out of the total 518 milk samples tested by trans-PCR, 24 (4.63%) samples detected as positive were comprised of 23 (5.53%) from bovines (414) including 21 (6.79%) of buffaloes (309) and 02 (1.90%) of cattle (105), and 01 (1.42%) from sheep (70) (Table 2). However, all the camel milk samples tested were found negative. None of the meat and egg samples screened by the PCR assay showed positivity for C. burnetii.
Table 1. Over all positivity of food samples by trans-PCR

<table>
<thead>
<tr>
<th>Type of food sample</th>
<th>Source/ Species</th>
<th>Samples Tested</th>
<th>Samples Positive</th>
<th>% Positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>Cattle, Buffalo, Sheep, Camel</td>
<td>518</td>
<td>24</td>
<td>4.63%</td>
</tr>
<tr>
<td>Meat</td>
<td>Goat, Hen</td>
<td>60</td>
<td>–</td>
<td>0.00%</td>
</tr>
<tr>
<td>Egg</td>
<td>Hen</td>
<td>13</td>
<td>–</td>
<td>0.00%</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>591</td>
<td>24</td>
<td>4.06%</td>
</tr>
</tbody>
</table>

Table 2. Screening of milk samples by trans-PCR

<table>
<thead>
<tr>
<th>Area of collection</th>
<th>Source / Species</th>
<th>Samples Tested</th>
<th>Samples Positive</th>
<th>% Positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm &amp; field cases, Maharashtra, Goa</td>
<td>Buffalo</td>
<td>309</td>
<td>21</td>
<td>6.79%</td>
</tr>
<tr>
<td>Farm &amp; field cases, Goa, Odisha, U.P.</td>
<td>Cattle</td>
<td>105</td>
<td>02</td>
<td>1.90%</td>
</tr>
<tr>
<td>Field cases, Rajasthan</td>
<td>Sheep</td>
<td>70</td>
<td>01</td>
<td>1.42%</td>
</tr>
<tr>
<td>NRCC, Bikaner, Rajasthan</td>
<td>Camel</td>
<td>34</td>
<td>–</td>
<td>0.00%</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>518</td>
<td>24</td>
<td>4.63%</td>
</tr>
</tbody>
</table>

DISCUSSION

Of late the detection of C. burnetii in bulk tank milk samples from dairy herds in USA (year 2002 to 2004) with over 94% positivity by Real time PCR has suggested that the pathogen might persist in the dairy animals, with minor variations as regards to temporal or regional environment (Kim et al., 2005). However, only few of the reported studies till the year 2006 have analyzed foodstuffs for the presence of C. burnetii employing the molecular tool of PCR (Fretz et al., 2007). Similarly, the study of screening foods of animal origin for occurrence of C. burnetii in India, especially by highly sensitive and specific molecular diagnostic tools is largely lacking, and in this context, our study appears to be the first of its kind in India.

An overall positivity of 4.63% milk samples (5.55% from bovines and 1.42% from ovines) for C. burnetii by trans-PCR observed in our study is comparable to earlier reported studies with a positivity of 4.7% of bovine milk samples in Switzerland by nested PCR (Fretz et al., 2007). 3.5% of 400 sheep milk samples by IMS-PCR in Turkey (Ongor et al., 2004); and 6.2% of 210 bovine milk samples by nested PCR assay in Iran (Rahimi et al., 2009). The relatively high positivity of bovine milk samples and low positivity of ovine milk samples for the pathogen observed in our study is in agreement with the reported high (over 94%) detection rate of pathogen in bovine bulk milk tank in USA (Kim et al., 2005) but a complete absence of C. burnetii in ovine bulk milk samples tested in Iran (Rahimi et al., 2009) and Switzerland (Fretz et al., 2007). These reports indicate that the bovine milk, even from apparently healthy animals may serve as significant sources of C. burnetii infection. Moreover, the relatively high positivity (5.55%) of bovine milk in India observed in the present study also corroborates with our previous findings where the prevalence of Q fever among bovines with a history of reproductive disorders was observed to be higher (12.78% in cattle, 16.66% in buffaloes) compared to small ruminants (11.04% in sheep, 6.13% in goats) (Vaidya et al., 2010). Moreover, the excretion of the pathogen in bovine milk has been reported to last as long as 13–32 months (Arricau-Bouvery and Rodelakis, 2005; Angelakis and Raoult, 2010). The present study indicate that the occurrence of C. burnetii in bovine milk in India might be common, and therefore, a large scale screening is needed for evaluation and implementation of necessary legal regulations with regard to coxiellosis in dairy herds.

Non-detection of C. burnetii in the meat samples observed in our study might be due to the absence of the pathogen in the animal population screened. The published reports on occurrence of C. burnetii in meat are largely lacking. Moreover, the detection of C. burnetii in meat and offal, even in the infected animals, seems to be low as the screening of meat, offal and animal byproducts destined for human consumption in Germany has revealed the pathogen in only 8% of musculature (meat) of Q fever infected cattle whereas other animal parts used for meat purposes were found less infected (Schaal, 1977).

Mass screening of eggs and their products in Toyama prefecture in Japan revealed 4.2% of eggs and 17.6% of mayonnaise specimens to be positive for C. burnetii by PCR (Tatsumi et al., 2006). In a recent study from Iran, PCR revealed positivity for C. burnetii in 1.5% of hen eggs and 7.7% duck eggs (Rahimi and Doosti, 2012). However, small number of eggs (13) screened by PCR in our study were found free from C. burnetii infection, which is similar to a reported Swiss study, wherein all the 304 shell eggs analyzed with nested PCR were found negative for C. burnetii (Fretz et al., 2007). However, the lack of association of egg samples with C. burnetii infection observed in our study on limited samples should not be considered as a measure of prevalence of C. burnetii in these widely consumed foods of animal origin, especially in the light of high prevalence of the pathogen in eggs from other parts of the world (Tatsumi et al., 2006; Rahimi and Doosti, 2012).

In conclusion, the Q fever infection in countries like India remains grossly under diagnosed and the exact burden of the disease largely remains unknown. The detection of C. burnetii DNA in the bovine and ovine milk indicate that dairy animals in India may serve as the potential source of the infection through their milk and represent a risk for other domestic livestock animals, especially in suckling calves, as well as human beings, especially in breast fed babies (Kumar et al., 1981) and those who consume raw or unpasteurized milk as the diet or sacred offerings in the temples, which has been reported to be the vehicle of zoonotic pathogens like Listeria monocytogenes (Sheela Mary and Shrinithivahashini, 2013). In the light of these observations, it is recommended that milk from dairy animals should be consumed only after pasteurization and it would be better to include dairy as well as other food animals in surveillance and monitoring programmes. Besides this, the risk assessment studies addressing the type of food animal involved, areas or regions affected are of prime importance to ascertain the exact burden of C. burnetii infection in foods of animal origin.

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