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ORIGINAL ARTICLE

Screening of fecal droppings of wild birds for coxiellosis by a duplex PCR targeting *Com1* and *IS1111* genes of *Coxiella burnetii*

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Abstract

Many domestic and wild mammals, birds and arthropods such as ticks are known to act as reservoirs of *Coxiella burnetii*, the obligate intracellular bacterium that causes the most contagious disease - Q fever. However, the association of wild birds with this pathogen and their possible role in its perpetuation and spread has not been studied much. In the present study, 21 fecal samples of wild birds were screened for *C. burnetii* infection by duplex PCR targeting the *Com1* and *IS1111* genes of *C. burnetii*. Out of the 21 fecal samples of wild birds screened, only one (4.76%) blood tinged faecal sample from a free roaming bird of unknown source revealed positivity for only *Com1* gene of *C. burnetii*, while all the remaining samples turned out negative for *Com1* and *IS1111* gene. The pathogen was isolated from this lone *Com1* gene positive sample by chick embryo inoculation method and reconfirmed using nested *Com1* gene PCR and sequencing. This study, which appears to be first of its kind in India, indicate the usefulness of the duplex PCR as a rapid and reliable molecular diagnostic tool for screening the samples, as it is capable of detecting either of the two specific genes of *C. burnetii* in a single PCR reaction, with an added advantage of *com1* gene targeted-PCR being applicable to serum. Moreover, the association of wild birds with *C. burnetii* infection observed in this study on a limited samples calls for a systematic study on a larger population of birds of different origin and types for elucidation and extent of their role in the epidemiology of coxiellosis in the nature, especially in this part of the world.

Keywords: Wild bird, duplex-PCR, *Com1*, *IS 1111*, India

Introduction

Q fever is the most contagious zoonotic disease caused by a highly infectious Gram-negative obligate intracellular bacterium - *Coxiella burnetii* (ILRI, 2012). It has remained endemic in many parts of the world with its confirmed prevalence in at least 51 countries including India (Marrie, 2003; Malik and Vaidya, 2005; Alberta Health, 2012). In recent times, the disease is emerging or re-emerging in many countries (Natale *et al.*, 2012). The unprecedented outbreak of Q fever in Netherland since 2007 has affected over 4000 human cases (Toman *et al.*, 2012, Roest *et al.*, 2012, Schets *et al.*, 2013) and led to the culling of 51,820 small ruminants besides leaving 17.5% of 517 culling workers seropositive to *C. burnetii* despite use of personal protective equipment (Whelan *et al.*, 2011). This has highlighted the public health impact and re-emergent nature of Q fever, in addition to the need for improved surveillance and control methods (Toman *et al.*, 2012). In humans disease is associated with a wide clinical spectrum ranging from asymptomatic or a self-

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limited disease that usually appears as mildly symptomatic seroconversion, undifferentiated fever, pneumonia, or hepatitis to chronic fatal endocarditis (Angelakis and Raoult, 2010).

Rapid and early diagnosis of the disease particularly in early acute stage is very important as it may help in appropriate antibiotic treatment, and thereby, a better prognosis for individuals suffering from Q fever. Of late, a PCR assay that specifically detects the transposon- based *IS1111* insertion sequence of *C. burnetii* (Trans-PCR) has been widely used for detection of *C. burnetii* in different clinical samples (Willems *et al.*, 1994; Ho *et al.*, 1996; Berri *et al.*, 2000; Rolain and Raoult, 2005; Vaidya *et al.*, 2008; 2010) and environment (Kersh *et al.*, 2010). However, it has been demonstrated that the *Com1* gene encoding a 27-kDa outer membrane protein (OMP) being highly conserved, sensitive and specific can be the genetic target for the detection of *C. burnetii* in clinical samples, particularly in early acute Q fever cases (Zhang *et al.*, 1998).

The detection of high numbers of *C. burnetii* cells in environment (Kersh *et al.*, 2010) and clinical samples, as well as the frequent association of this pathogen with cases of reproductive disorders in humans (Vaidya *et al.*, 2008), animals (Vaidya *et al.*, 2010) by trans-PCR has amply indicated that Q fever remains underdiagnosed and underreported in many countries including USA (Kersh *et al.*, 2010) and India (Malik and Vaidya, 2005; Malik *et al.*, 2009; Das, 2010; Malik *et al.*, 2012). Multiple hosts including domestic and wild mammals, birds and arthropods such as ticks can serve as a natural reservoir of the *C. burnetii* (Maurin and Raoult, 1999; Komiya *et al.*, 2003; Ruiz-Fons *et al.*, 2008; Agerholm, 2013). The infection is transmitted from animals and birds to human beings, mainly through inhalation of contaminated aerosols from animal birth materials or contaminated dusts containing *C. burnetii* shed from infected animals and birds. The infection in these animals and birds is mostly subclinical or in-apparent (Tissot-Dupont *et al.*, 1999; 2004; Guatteo *et al.*, 2011). However, in the first reported bird-borne human outbreak of Q fever 5 persons developed Q fever pneumonia due to exposure to contaminated pigeon feces (Stein and Raoult, 1999). Very few studies have been carried out on the distribution and incidence of *C. burnetii* infection in birds, particularly of wild nature, accordingly, potential threat from these infected birds to humans, domestic animals and other domestic as well as wild birds largely remains unknown.

The aim of present study was to screen faecal samples of some wild birds in India for presence of the pathogen by a duplex PCR targeting *Com1* and *IS1111* genes of *C. burnetii*, with a view to assess the possible role of these birds in the maintenance and spread of this pathogen.

Materials and Methods

Samples: A total of 21 fecal samples were collected from 19 wild birds from Nandankanan Biological Park, Bhubaneswar, India and 02 blood tinged fecal droppings (found on the front glass of car) from unknown free roaming birds in the IVRI campus, Izatnagar, Bareilly, India. All the samples were collected aseptically either with help of swab(s) in PBS or directly into sterile vials and kept at 4^0c for further processing.

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

DNA extraction: Before processing for DNA extraction, all the samples were treated overnight with proteinase K. The DNA was then extracted from fecal samples of wild birds by using QIAamp DNA Stool Kit as per the instructions provided by manufacturer (Qiagen, USA). The DNA was quantified using nanodrop NG 100 (Thermo Scientific, USA).

PCR assay: All the primers used in present study were designed based on earlier published literature (Zhang *et al.*, 1998; Marmion *et al.*, 2005; Lorenz *et al.*, 1998). The novel duplex PCR targeting *Com1* and *IS 1111* genes of *C.*

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burnetii was standardized for the first time in this study. The primers were synthesized from a commercial source (Sigma Aldrich, Ltd.). The sequence of the primers used in the duplex PCR are Com-1 forward (5'-AGT AGA AGC ATC CCA AGC ATT G-3') and Com-1 reverse (5'-TGC CTG CTA GCT GTA ACG ATT G-3') targeting *Com1* gene corresponding to 27kDa OMP and trans forward (5'-GTA ACG ATG CGC AGG CGAT-3') and trans reverse (5'-CCA CCG CTT CGC TCG CTA-3') targeting the transposon-like repetitive element *IS1111* of *C. burnetii*. The primers specifically amplified 501bp of *Com*1 gene and 243 bp fragments of *IS1111* element of *C. burnetii*, respectively. The sequences of nested primer used for confirmation of *Com*1 gene amplification (501 bp) are Com-2 forward (5'-GAA GCG CAA CAA GAA GAA CAC-3') and Com-2 reverse(5'-TTG GAA GTT ATC ACG CAG TTG-3') which yielded a amplified product of 438 bp.

The DNA of *C. burnetii* RSA 493 Nine Mile 1 strain was used as a positive control and nuclease free water as negative control. The PCR reaction mixture (25 µl) included 2.5 µl of 10 X PCR buffer (100 mM Tris–HCl buffer, pH 8.3 containing 500 mM KCl, 15 mM $MgCl_2$ and 0.01% gelatin), 200 mM dNTP mix, 3 Mm $MgCl_2$, 2 mM each primer, 0.5 unit of Taq DNA polymerase, 5 µl of DNA extract and sterilized milliQ water to make up the reaction volume. The DNA amplification reaction was performed in Master Cycler Gradient Thermocycler (Eppendorf, Germany). The cycling conditions included an initial denaturation at 95°C for 2 min followed by 35 cycles, each consisting of 95°C for 30 s, 55-65°C for 1 min and 72°C for 1 min. The final stage of the reaction included an extension of 10 min at 72°C. The resultant PCR products were analyzed by agarose gel electrophoresis (1.5%; low-melting temperature agarose l), stained with ethidium bromide and visualized by a UV transilluminator (UVP Gel Seq Software, UK). The standardized duplex-PCR assay was used in this study for screening faecal samples of wild and free-roaming birds for *C. burnetii* DNA.

Results and Discussion

The objectives of this study were to determine the presence of *C. burnetii* in faecal droppings of wild birds and to optimize available detection methods. Several studies have reported serological and molecular evidences of *C. burnetii* infection in wild species suggesting their involvement in the wild cycle of *C. burnetii* in nature around the world (Enright *et al.*, 1971a; 1971b; Giovannini *et al.*, 1988; Ejercito *et al.*, 1993; Barandika *et al.*, 2007; Ruiz-Fons *et al.*, 2008; Astobiza *et al.*, 2011). Serological methods are the best suited for getting an indirect evidence of coxiellosis in birds on account of expected long persistence of detectable circulating antibodies against *C. burnetii*. However, sera collection from wild birds not only remains a very difficult task but also requires legal permissions, which are practically hard to get. Moreover, these serological tests have some limitations. Antibodies can not be detected during the early stage of the infection, and it is difficult to discriminate between current and past infection by a test with a single serum sample, because antibodies often persist after the organisms disappear from the blood. Thus, serological tests offer only a retrospective diagnosis and are useless for the treatment of the affected patients (Zhang *et al.*, 1998). Serological techniques are less suitable for direct transmission and source-finding studies for *C. burnetii* infection due to the delayed detection window for serological tests (de Bruin and van Rotterdam, 2011). It becomes all the more necessary to develop and standardize more sensitive and specific diagnostic molecular tests for pathogen detection in clinical samples in view of the high infectivity, stability, ease of dissemination, and intermittent shedding of *C. burnetii* (Malik *et al.*, 2009; 2012).

In India, studies employing different modern diagnostic tools namely trans-PCR and real-time trans-PCR have shown the prevalence of Q fever in 21.62 % of human cases with spontaneous abortions (Vaidya *et al.*, 2008) and 11.05 % of domestic animals with reproductive disorders (Vaidya *et al.*, 2010). However,

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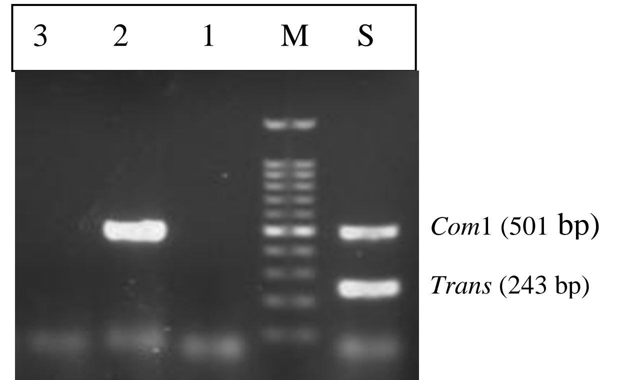
studies in particular concerning to wild and migratory birds in the country are scanty. Therefore, the aim of this study was to investigate the presence of the Q fever pathogen in the wild birds in India by means of polymerase chain reaction (PCR) targeting *Com1* and *IS1111* genes of *C. burnetii* with the purpose of assessing the importance of wild birds in maintaining the cycle of Q fever in nature. Hence, in the present study a duplex PCR was employed to screen the faecal droppings of these birds. To the best of our knowledge, the diagnostic approach employed in our study is the first to use two different sets of primers in a single PCR, i.e., targeting *com1*gene for OMP, encoding a 27-kDa protein and *IS1111* gene for the detection of *C. burnetii* DNA in bird fecal droppings.

In view of high sensitivity and specificity, the *IS1111* repetitive element is the best known target gene and presently being used widely for the detection of *C. burnetii* in patients with active *C. burnetii* infections. However, the *Com1* gene sequence was also chosen for the target of PCR amplification, as this gene has been reported to be highly conserved among 21 strains of *C. burnetii* from various clinical and geographical sources (Zhang *et al.*, 1998), and the PCR based on it (Com1-PCR) was found to be highly sensitive and specific for the detection of *C. burnetii* in clinical samples including serum samples (Marmion *et al.*, 2005). Besides this, Real-time PCR targeting the *Com1*, *IS1111* and *Icd* genes has been employed for detection of *C. burnetii* in both the ticks and whole blood of a variety of native Australian marsupials (Cooper *et al.*, 2013).

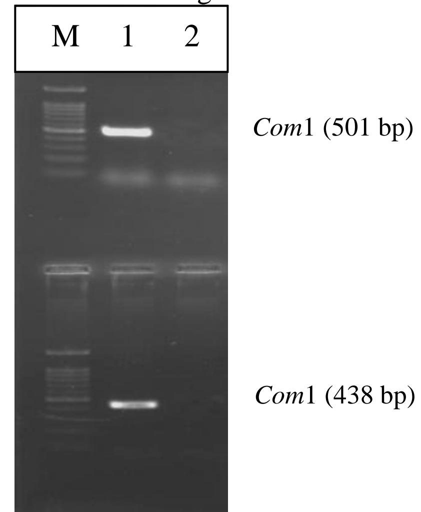
In the present study, 21 fecal samples of wild birds were screened for *C. burnetii* infection by duplex PCR. Out of the 21 fecal samples of wild birds screened, only one (4.76%) blood tinged faecal sample from a free roaming bird of unknown source revealed positivity for 501bp product corresponding to *Com1* gene of *C. burnetii*, while the same positive sample as well as all the other samples turned out to negative for *IS1111* gene (Fig 1).

Fig 1: Duplex PCR targeting *Com1* and *IS1111* genes of *Coxiella burnetii.*



Lane S: Standard *C. burnetii* DNA
Lane M: 100 bp DNA Marker
Lane 1 : Negative control
Lane 2 : Positive faecal sample of a free roaming bird
Lane 3 : Negative sample

Fig 2: Com1 nested PCR of positive faecal sample of a free roaming bird.



Lane M-100 bp DNA Marker
Lane 1: Positive faecal sample of a free roaming bird
Lane 2: Negative control

The pathogen was also isolated from this lone blood tinged faecal sample that showed positivity for *Com1* gene in duplex PCR, by chick embryo inoculation method, and was reconfirmed using nested PCR targeting *Com1*

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gene (Fig 2) as shown above as well as by sequencing of Com1-PCR product.

The detection of *C. burnetii* DNA in blood tinged fecal dropping of a free roaming wild bird by duplex PCR method observed in our study with positivity for *Com1* gene only but negativity for *IS1111* gene might be attributed to the possibility of this detected and isolated *C. burnetii* strain being a *IS1111*-deficient isolate, similar to that observed in an earlier report (Marmion *et al.*, 2005). Moreover, the PCR-positive fecal sample in our study was blood-tinged, and therefore, might have revealed positivity for *Com1* gene that corresponds to Com1 protein, a 27-kDa immuno-reactive outer membrane protein (OMP) of *C. burnetii* commonly found in the blood or serum (Zhang *et al.*, 1998). However, successful isolation of the pathogen from *Com1*-PCR positive blood tinged bird faecal drooping and its confirmation using nested primer of *Com1* gene and sequencing results provide the scientific support to confirm our findings that the duplex PCR used in this study was specific and free from any contamination of positive control amplicon. Our results are in agreement with the observations reported by other workers that the Com1-PCR is highly specific and sensitive for the detection of *C. burnetii* in clinical samples including serum (Zhang *et al.*, 1998) and may be used along with trans-PCR (Marmion *et al.*, 2005).

This study, which appears to be first of its kind in India, indicate the usefulness of the duplex PCR as a rapid and reliable molecular diagnostic tool for screening the samples, with an added advantage of *com1* gene targeted-PCR being applicable to serum samples. However, the present study was done on limited numbers of samples which are insufficient to draw a final conclusion for ascertaining the exact burden of *C. burnetii* in wild birds in the country. Therefore, the association of wild birds with *C. burnetii* infection observed in this study on a limited samples calls for a systematic study on a larger population of birds of different origin and types for elucidation and extent of their role in the epidemiology of coxiellosis in the nature, especially in this part of the world.

Conclusion

In conclusion, the Q fever infection grossly remains under diagnosed and we do not know the exact burden of the disease. The detection of *C. burnetii* DNA in the blood-tinged faeces of a free roaming wild bird whose source and identification was not known, and isolation of the pathogen from it indicate that wild birds may serve as the potential source for the infection and represent a risk for other domestic and wild livestock animals and birds as well as human beings, especially those share their habitats, niche and migration areas. In the light of these observations, it would be better to include wild as well as migratory birds in surveillance and monitoring programs targeting livestock and humans. It would greatly help in the risk assessment of this important zoonotic infection for humans and other domestic animals based on the identification and monitoring of the species of birds involved, areas or regions affected, and their shedding patterns.

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