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 lome 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 Nederland 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-
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
asceptically either with help of swab(s) in PBS or
directly into sterile vials and kept at 4°C 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 protease 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 IS1111 genes of C.
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 TCT CGC TCG GGA-3’) targeting the transposon-like repetitive element IS1111 of C. burnetii. The primers specifically amplified 501bp of Com1 gene and 243 bp fragments of IS1111 element of C. burnetii, respectively. The sequences of nested primer used for confirmation of Com1 gene amplification (501 bp) are Com-2 forward (5’-GAA CCG 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 MgCl2 and 0.01% gelatin), 200 mM dNTP mix, 3 Mm MgCl2, 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,
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). 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.
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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References


Das DP (2010). Detection of *Coxiella burnetii* infection in man, animals and ticks by different diagnostic tests employed for Q fever. MVSc. Thesis. Indian Veterinary Research Institute, Izatnagar, India.


Malik SVS, Rawool DB and Das DP (2012). Recent advances for control and prevention of zoonotic diseases. In: Training Manual on Recent Advances in Animal Disease Diagnosis and their Treatment, T. Dutt, M.C. Sharma and R. Tiwari (Eds), Jan 16-23, Joint Directorate of
Das et al.……………PCR based screening of fecal droppings of wild birds for coxiellosis

Extension Education, Indian Veterinary Research Institute, Izatnagar, India, pp.69-84.


