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RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

Isolation of *Coxiella burnetii* from aborted fetuses of bovine and buffalo and its identification through nested and real-time PCR assays

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Abstract

The epidemiology and prevalence of *Coxiella burnetii* in aborted fetus of bovine and buffalo in various seasons and partums is unknown. This study was designed to find the prevalence of *C. burnetii* in bovine and buffalo in different areas, season, months and post-partum period through nested and real time polymerase chain reaction (PCR). Our results indicated that nested PCR 14 (4.68%) and 11 (4.29%) were positive for presence of *C. burnetii*. Real-time PCR showed that 19 (6.35%) and 13 (5.07%) of samples were positive for *C. burnetii* in aborted fetus of bovine and buffalo. Gilan (8.57%) had the highest and Boshehr (3.44%) Kerman and Ilam (0.0%) had the lowest prevalence of *C. burnetii* in aborted bovine and buffalo fetus. Our results indicated that bovine is more sensitive than buffalo to *C. burnetii*'s abortion (6.35% versus 5.07%). Our results showed that the prevalence of *C. burnetii* in winter (December-February), spring (March-May), summer (Jun-August) and autumn (September-November) of the year were 10.52%, 84.26%, 5.26% and 0.0% in cows and 23.07%, 69.23%, 0.0% and 7.69% in buffalo, respectively. From the total 19 bovine and 13 buffalos aborted fetuses, 14 (73.68%) and 8(61.53%) were positive from first partum respectively. No *C. burnetii* was positive in bovine and only 1 (7.69%) isolate was positive from buffalo in Fifth and last five partum.

Keywords: *Coxiella burnetii*; aborted fetuses; seasonal pattern; various partum; nested PCR; real-time PCR

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Introduction

Query fever (Q-fever) or coxiellosis is a zoonotic bacterial disease caused by an obligate intracellular Gram-negative *Coxiella burnetii* having worldwide distribution (Raoult et al., 2005). A wide variety of animals such as primates, ruminants, cats, dogs, small mammals, wild rodents, big game wildlife and non-mammals such as birds, amphibians, fish, reptiles and ticks may be infected by *C. burnetii* (Parker et al., 2006). In the majority of cases, domestic and companion animals like ruminants and pets are the most important source for human infection. To the author's knowledge, almost in many cases ovine and caprine are the most prevalent hosts of *C. burnetii* and in some conditions, when ruminants are kept in close contact with infected ovine and caprine.

The uterus is primary site of infection in the chronic phase of *C. burnetii*. Shedding of *C. burnetii* in the environment occurs mainly during parturition by birth products, particularly the placenta but may also be shed via vaginal mucus and semen (Rahimi et al., 2010). Some study showed that high concentration of *C. burnetii* is found in the placenta of infected animals (Foley et al., 2003).

Due to the high economic losses caused by *C. burnetii*, it is need of the time to use accurate and sensitive diagnostic methods for rapid identification and elimination of persistent carriers in the herds. Among all diagnostic techniques (culture, serology etc.),

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molecular methods for detection of *C. burnetii* due to high sensitivity (Guatteo et al., 2007), specificity (Ongor et al., 2004), safety (Berri et al., 2003) and speed (Spyridaki et al., 2002) has been recommended. Coxiellosis occurs during late pregnancy (about 15 days before term) which leads to abortion in small ruminants and stillbirth in cattle (Fournier et al., 1998). Several studies showed that this bacterium can cause abortion in small ruminants (Berri et al., 2002; Dehkordi, 2011) but in our knowledge, there is no data about occurrence of abortion caused by *C. burnetii* in bovine and buffalo fetus. On the other hand, although there have been a significant number of abortions in domestic animals in Iran. There is no report on reproductive problems and the causes of abortion in ruminants in Iran.

Therefore, the two fold purpose of this study was to detect *C. burnetii* in aborted fetus of bovine and buffalo through nested and real-time PCR in various seasons and partum.

Materials and Methods

Samples collection

From January 2010 to January 2011 in various seasons of the year, a total of 299 bovine and 256 buffalo aborted fetus were collected from 108 dairy herds of ten provinces of different parts of Iran (Table I). These samples were collected randomly from first, second, third, forth and fifth partum bovine and buffalo under sterile conditions and were immediately transported to the Biotechnology Research Centre of Islamic Azad University of Shahrekord in a cooler with ice packs. All abomasal content samples were kept at -20°C until processing.

Cell culture

Isolation of *C. burnetii* was performed using Buffalo Green Monkey (BGM) cell culture. Cells were propagated in 25 cm^2 plastic flasks with an Ultra Culture medium (BioWitthaker, Walkersville, Maryland, USA) without supplements. Material of each sample (1 g) was homogenized using sterile mortar, sand and cell culture medium. The supernatants were filtered through membrane filters (Minisart™ Sartorius, Göttingen, Germany) with pore diameters of 0.2 µm. A volume of 0.5 ml homogenate per flask was inoculated. Cell culture was examined weekly by phase-contrast microscopy for inclusion bodies.

DNA extraction

C. burnetii DNA was extracted using a genomic DNA purification kit (Invitrogen, Paisley, U.K.) according to the manufacturer's instruction and the total DNA was measured at 260 nm optical density with the method described by Sambrook and Russell (2001).

Nested PCR

All oligonucleotide primers were obtained from a commercial source (Cinna Gen, Iran). The nested PCR assay used to screen *C. burnetii* in aborted fetuses was designed from the nucleotide sequence of the com1 gene encoding a 27-kD outer membrane protein (OMP) as previously described (Zhang et al., 1998) and the amplification was carried according to the method described elsewhere (Fretz et al., 2007). For the nested PCR assay with primers OMP1-OMP2 and OMP3-OMP4, the first amplification was performed in a total volume of 25 l containing 5 l of DNA sample, 0.5 mm $MgCl_2$, 0.2 mM (each) dNTPs, 1 m primer OMP1, 1 m primer OMP2, and 0.5 U/reaction of Taq DNA polymerase (Promega, Madison, WI). The PCR assay was performed at 94 for 4 min and then for 30 cycles of 94 for 1 min, 56 for 1 min, and 72 for 1 min in a DNA thermal cycler (ASTEC, Fukuoka, Japan). In the second amplification, the reaction was performed in a total volume of 25 l containing 2 l of DNA sample, 0.5 mm $MgCl_2$, 0.2 mm (each) dNTPs, 0.82 m primer OMP3, 0.8 lm primer OMP4, and 0.5 U/reaction of Taq DNA polymerase (Promega, Madison, WI). The PCR assay was performed for 4 min and then for 30 cycles of 94 for 1 min, 57for 1 min and 72 for 1 min.

Table 1: Isolation of *C. burnetii* from bovine and buffaloes in ten provinces of Iran using nested and real-time PCR

Provinces	Number of samples		Nested PCR (%)		Real-Time PCR (%)	
	Bovine	Buffalo	Bovine	Buffalo	Bovine	Buffalo
Boshehr	29	27	1 (3.44)	1 (3.7)	1 (3.44)	1 (3.7)
Hormozgan	40	31	1(2.5)	1(3.22)	2(5)	2(6.45)
Khozestan	33	30	1(3.03)	1(3.33)	2(6.06)	2(6.66)
Khorasan	26	20	1(3.84)	-(-)	1(3.84)	1(5)
Sistan va Balochestan	27	22	3(4.22)	2(2.66)	4(5.63)	2(2.66)
Kerman	21	24	-(-)	1(4.16)	1(4.76)	-(-)
Gilan	38	35	2(5.36)	3(8.57)	3(7.89)	3(8.57)
Fars	34	25	2(5.88)	1(4)	2(5.88)	1(4)
Kordestan	31	22	2(6.45)	1(4.54)	2(6.45)	1(4.54)
Ilam	20	20	1(5)	-(-)	1(5)	-(-)
Total	299	256	14(4.68)	11(4.29)	19(6.35)	13(5.07)

Values in parenthesis indicates percentage

Gel electrophoresis

The PCR-amplified products (OMP1- OMP2: 501 bp; OMP3-OMP4: 438 bp) were examined by electrophoresis in a 1.5% agarose gel, stained with a 1% solution of ethidium bromide and examined under UV illumination. In this study, *C. burnetii* DNA (Serial Number: 3154; Genekam Biotechnology AG, Duisburg, Germany) and DNase free water were used as the positive and negative controls, respectively.

Real-Time PCR

The primer set consisted of primers trans-f (5′-GGGTAAAACGGTGGAACA ACA-3′) and trans-r (5′-ACAACCCCCGAATCTCATTG-3′). The internal probe trans-p (5′-AACGATCGCGTATCTTTAACA GCGCTTG-3′) was labeled with the reporter dye5-carboxyfluoroscein (FAM) on the 5′ end and the quencher dye N′, N′, N′, N′-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3′ end.

Each sample was tested using the commercial kit (targeting the repetitive transposon-like region of *C. burnetii*), LSI Taqvet *C. burnetii* (Laboratoire Service International, Lissieu, France) assay, according to the manufacturer's instructions. The negative control sample used was DNase RNase free. The external positive control used was a solution containing 10^5 *C. burnetii*/mL (provided by UR INRA IASP, Nouzilly, France). DNA extraction was performed directly from 200 µL of abomasal contents. All Real time PCRs reactions were carried out using a RotorGene 6000 instrument (Corbett Research). For positive samples (having a typical amplification curve), the results are given in Ct (cycle threshold) values. Only the samples presenting a typical amplification curve with a Ct below 40 were considered positive.

Sensitivity & specificity of nested and real-time PCR

The sensitivity and specificity of each test was determined using the formula as follows:

Sensitivity: True positive/true positive + false negative x 100

Specificity: True negative/true negative + positive x 100

Statistical Analysis

Data were transferred to Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA) for analysis. Using SPSS 18.0 statistical software (SPSS Inc., Chicago, IL, USA), ANOVA was performed and differences were considered significant at values of $P<0.05$.

Results

Based on nested PCR 14 (4.68%) and 11 (4.29%) were positive for presence of *C. burnetii*. Real-time PCR showed that 19 (6.35%) and 13 (5.07%) of samples were positive for *C. burnetii* in aborted fetus of bovine and buffalo (Table 1). So this study showed real-time PCR had a higher accuracy, sensitivity and specificity than nested PCR to detection of *C. burnetii* in aborted bovine and buffalo fetuses.

In the present study, Gilan (8.57%) had the highest and Boshehr (3.44%) Kerman and Ilam (0.0%) had the lowest prevalence of *C. burnetii* in aborted bovine and buffalo fetus (Table 2). Our results indicated that bovine is more sensitive than buffalo to *C. burnetii*'s abortion (6.35% versus 5.07%).

Our results showed that the prevalence of *C. burnetii* in winter (December-February), spring (March-May), summer (Jun-August) and autumn (September-November) of the year were 10.52%, 84.26%, 5.26% and 0.0% in cows and 23.07%, 69.23%, 0.0% and 7.69% in buffalo, respectively (Table 2). Therefore, our study showed that *C. burnetii* abortion in both bovine and buffalo species occurred mainly in spring (March-May) season (Table 2).

From the total 19 bovine and 13 buffalos aborted fetuses, 14 (73.68%) and 8(61.53%) were positive from first partum respectively. No *C. burnetii* was positive in bovine and only 1 (7.69%) isolate was positive from buffalo in fifth and last five partum (Table 3).

Discussion

Our study showed that application of real-time PCR as a sensitive, specific and safe assay for detection of *C. burnetii* in aborted fetus can be effective for rapid diagnosis of abortion in all animals. The real-time PCR assay used in this study is more specific, sensitive and faster than nested PCR. In addition, the real-time PCR assay has some advantages compared to the nested PCR; it is an important diagnostic tool yielding reliable and reproducible results, does not require post-PCR analysis (gel electrophoresis, hybridization), and the risk of cross contamination is limited than nested PCR method but the real-time PCR is more expensive than nested PCR. On the other hand, our experience indicated that the nested PCR for detecting *C. burnetii* is time-consuming and labor-intensive than real-time PCR assay.

The real-time PCR assay used in this study can simplify the procedure by testing presumptive *C. burnetii* genome taken directly from abomasal contents of aborted cows and buffaloes fetus. Using the LSI Taqvet assay offers specificity higher than that of gel electrophoresis and finally this real-time PCR can substantially decrease the risk of carry over contamination.

Previous studies suggest molecular methods for detection of *C. burnetii* in clinical samples for high

Table 2: Distribution of *C. burnetii* in aborted bovine and buffalo fetuses in different areas and months of the year

Provinces	No. of positive samples with real-time PCR (%)		December-February (%)		March-May (%)		Jun-August (%)		September-November (%)	
	Bovine	Buffalo	Bovine	Buffalo	Bovine	Buffalo	Bovine	Buffalo	Bovine	Buffalo
Boshehr	1(3.44)	1(3.7)	-	-	1	1	-	-	-	-
Hormozgan	2(5)	2(6.45)	-	1	2	1	-	-	-	-
Khozestan	2(6.06)	2(6.66)	-	-	2	2	-	-	-	-
Khorasan	1(3.84)	1(5)	-	-	1	1	-	-	-	-
Sistan Va Balochestan	4(5.63)	2(2.66)	1	1	3	1	-	-	-	-
Kerman	1(4.76)	-(-)	-	-	1	-	-	-	-	-
Gilan	3(7.89)	3(8.57)	1	1	2	1	-	-	-	1
Fars	2(5.88)	1(4)	-	-	2	1	-	-	-	-
Kordestan	2(6.45)	1(4.54)	-	-	1	1	1	-	-	-
Ilam	1(5)	-(-)	-	-	1	-	-	-	-	-
Total	19(6.35)	13(5.07)	2(10.52)	3(23.07)	16(84.21)	9(69.23)	1(5.26)	-(-)	-(-)	1(7.69)

Table 3: Distribution of *C. burnetii* in various partums of bovine and buffalo aborted fetuses

Species	No. positive samples	First Partum (%)	Second Partum (%)	Third Partum (%)	Fourth Partum (%)	Fifth and top five partum (%)
Bovine	19	14(73.68)	3(15.78%)	1(5.26%)	1(5.26%)	-
Buffalo	13	8(61.53)	2(15.38)	1(7.69)	1(7.69)	1(7.69)
Total	32	22(68.75)	5(15.62)	2(6.25)	2(6.25)	1(3.12)

specificity, sensitivity and accuracy (Lorenz et al., 1998; Mossienko et al., 2003). PCR has been developed for the detection of *C. burnetii* for a wide variety of samples such as mice (Mossienko et al., 2003), milk (Rahimi et al., 2010), aborted fetus (Safarpoor Dehkordi, 2011), semen (Milazzo et al., 2001), tick (Nourollahi Fard and Khalili, 2011), blood (Lorenz et al., 1998), liver (Lorenz et al., 1998), feces (Berri et al., 2000), spleen (Lorenz et al., 1998), vaginal secretion (Cairns et al., 2007) placenta (Lorenz et al., 1998) and urine (Vaidya et al., 2008).

There are limited reports on prevalence of *C. burnetii* in aborted bovine and buffalo fetus and the current study for the first time presented coherent information about the direct detection and isolation of this bacterium in aborted bovine and buffalo fetus in various seasons and partum. In the current study, it is difficult to deny that dairy cattle with reproductive disorders would be one of the important reservoirs of *C. burnetii* (To et al., 1998).

Although a few studies have been conducted on Q-fever in Iran in some 50 years ago but in the past few years, various studies showed that *C. burnetii* is endemic in Iran (Rahimi et al., 2010, Safarpoor Dehkordi, 2011, Nourollahi Fard and Khalili, 2011, Khalili et al., 2011, Abbasi et al., 2011). Previous study suggested that *C. burnetii* can easily be transmitted to the bovine and buffaloes that are in close contact with infected ovine and caprine (Rahimi and Doosti, 2012).

In another study, 100 aborted fetus or stillborn calves were tested for presence of *C. burnetii* and its results showed that four placentas (seven to nine months gestation) were positive (Muskens et al., 2011). A recent study in Italy on buffaloes showed that 17.5% fetus of buffaloes were positive for presence of *C. burnetii* (Perugini et al., 2009). In recent study, in Cyprus, 37% samples collected from aborted ruminants were positive for *C. burnetii* (Cantas et al., 2011) and it was concluded that presence of ticks and carnivores in farm were the risk factors. Furthermore, the highest occurrence of *C. burnetii* was experienced in October which declined gradually to the lowest in December while our study showed that 84.36 and 69.23% of aborted cases were found in between March to May in bovine and buffaloes respectively. In addition, the seasonal variation of C. *burnetii* has previously been documented in cattle in Japan (Yanase et al., 1997) and showed that the positive cases in cows were significantly high in winter and decreased in summer. A study on human showed that seasonality occurrence of *C. burnetii* has been seen in spring following the outdoor lambing of sheep in southern Germany (Hellenbrand et al., 2001).

Our study showed that the majority of cases (68.75%) were found in first partum. We suggest that high stress of parturition and therefore weak immunity are the main reasons for this event.

Studies showed that *C. burnetii* has the ability to shed in feces, urine, milk, birth fluids and placenta (Guatteo et al., 2006) and testing animal for the presence of bacteria for one route can lead to misclassify the status of the animal. The reason of higher incidence of *C. burnetii* in bovine may be due to

close contact of these animals with infected ovine and caprine than buffalo (personal observation).

Our results showed that the majority of abortions caused by *C. burnetii* occurred from March to May and to the authors knowledge this period is the time of lambing. Therefore, probably the main cause of abortion in cows and buffalo herds in Iran may be the close contact of these animals with infected ovine and caprine during lambing period. Studies showed that ewes shed *C. burnetii* more and longer in vaginal discharges than goats, and can shed bacteria at subsequent pregnancies (Berri et al., 2003). Therefore, contact with infected ewes especially in lambing may have caused abortion in bovine and buffaloes.

The higher incidence of *C. burnetii* in aborted bovine and buffalo fetus may be due to the hot and humid environment of the Gilan province. To the author's knowledge, in addition to heat stress and humidity (which favour the growth) ticks might have caused the high prevalence of abortions.

Several studies from the United Kingdom, France, Spain, Italy, Israel, Germany, Canada and Greece (Angelakis and Raoult, 2010) indicate that Q-fever is a public health problem around the world. Since disease has been reported from some countries neighboring Iran such as Turkey (Kennermanm et al., 2010), Oman (Scrimgeour et al. 2003,), Iraq (Faix et al., 2008), United Arab Emirates (Afzal and Sakkir, 1994), Pakistan (Tariq et al., 2004) and Saudi Arabia (Hussein et al., 2012).

Conclusions

This study revealed that real-time PCR for detection of *C. burnetii* gives better results than nested PCR. Second, buffaloes are more resistance to *C. burnetii*'s abortion than cows. Third, *C. burnetii* has seasonal infection pattern in Iran and its incidence was more during March to May. Finally, *C. burnetii* is more prevalent during first partum.

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