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Prevalence Study of *Coxiella burnetii* in Aborted Ovine and Caprine Fetuses by Evaluation of Nested and Real-Time PCR Assays

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Abstract: Problem statement: Q fever is a ubiquitous zoonosis caused by *Coxiella burnetii*, an obligate intracellular rickettsial organism that caused abortion and stillbirth in ruminants. **Approach:** The prevalence of *Coxiella burnetii* in Iran is essentially unknown. Its traditional diagnosis is based on culture, serology and conventional PCR. In this present study, for more sensitive and accurate detection and prevalence's determination of *Coxiella burnetii* in aborted Ovine and Caprine fetuses, the nested and real-time PCR methods are recommended. **Results:** About 98 (12.53%) and 122 (16.39%) out of 782 and 744 Ovine and Caprine aborted fetuses, were positive for presence of *Coxiella burnetii* by nested PCR, respectively. After LSI Taqvet *Coxiella burnetii* real-time PCR, it was recognized that 121 (15.47%) and 152 (20.43%) samples were positive for *Coxiella burnetii* in Ovine and Caprine aborted fetuses, respectively. Results indicated that the real-time PCR was 7 times more sensitive than the nested PCR. Statistical analysis showed significant differences about $P<0.01$ between presence of *Coxiella burnetii* in aborted Ovine and Caprine fetuses by both nested and real-time PCR assays and $P<0.05$ between ability of nested and real-time PCR for detection of *Coxiella burnetii*. The Ct values which obtained from real-time PCR had significant differences about $P<0.01$ for presence of *Coxiella burnetii* between aborted Ovine and Caprine fetuses. Our results indicated that Caprine is more sensitive than Ovine to *Coxiella burnetii*'s abortion Khozestan and Gilan have the highest and Khorasan and Sistan va Baluchistan provinces have the lowest prevalence of *Coxiella burnetii*, respectively. **Conclusion:** To our knowledge, this study is the first prevalence report of direct identification of *Coxiella burnetii* in aborted Ovine and Caprine fetuses by evaluation of nested and real-time PCR assays in Iran. This study showed that the nested PCR for detecting *Coxiella burnetii* are technically time-consuming and labor-intensive.

Key words: *Coxiella burnetii*, aborted fetuses, nested PCR, real-time PCR

INTRODUCTION

Coxiella burnetii (*C. burnetii*) is a strict fastidious obligate intracellular Gram-negativebacteriumsimilar to rickettsia, which is a causative agent of important ubiquitous worldwide zoonotic infectious disease named coxiellosis or query fever (Q fever) (Raoult *et al*., 2005). Disease can transmitted between animals and human by reservoirsinclude several species of mammals, birds and arthropods, ticks, mites, fleas, lice and flies but domestic and companion animals such as Bovine, Ovine, Caprine and Pets are the most important sources of human infection (Dupuis *et al*., 1987; Rauch *et al*., 1987; Hatchette *et al*., 2001). Human infections have been reported mainly in persons handling infected animals and their products (Armengaud *et al*., 1997).

Cattle and small ruminant, when infected, shed the desiccation-resistant organisms in urine, feces, milk and, especially, in material getting out during abortion or parturition (Arricau-Bouvery and Rodolakis, 2005; Fournier *et al*., 1998). High concentrations of *C. burnetii* are found in the placentas of infected animals.

Coxiellosis occurs during late pregnancy (about 15 days before term) and leading to abortion in small ruminants and stillbirth in cattle (Russo and Malo, 1981).

Because *C. burnetii* is highly resistant to physical as well as chemical agents, it is difficult to control once it is disseminated in the environment.

Therefore *C. burnetii* with these economic losses, need to accurate and sensitive diagnostic methods for rapid identification and elimination of persistent carriers in the herds.

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There are various methods for diagnosis of *C. burnetii* such as culture, serological and molecular methods. Culture method require a living host and is both time consuming and hazardous.

The diagnosis of *C. burnetii* by serological responses, which can be unspecific and unsensitive due to cross-reaction (Rousset *et al*., 2009) or subsensitive reactionsin samples from areas with a low or subclinical prevalence of Coxiellosis. In addition serological diagnostic methods are retrospective due to the time-frame for sero-conversion (3-4 weeks post infection), rendering them useless for timely treatment. In the other hand antibodies against *C. burnetii* may not be appear until late into the course of the disease and it is often difficult to make an early diagnosis based solely on serology for animal which present early. Since early diagnosis would be helpful for treatment of animals. In these years DNA-based methods such as PCR, nested PCR and real-time PCR as safe and useful methods have been successfully used for detection of *C. burnetii* in clinical specimens (Berri *et al*., 2000; 2001; Kato *et al*., 1998; Klee *et al*., 2006). The ability to detect and quantify *C. burnetii* DNA by nested and real-time PCR has dramatically enhanced diagnostic and study approaches.

Although there have been a significant number of abortions in domestic animals in Iran, there is no study on reproductive problems and the causes of abortions in ruminants in Iran in the literature so far.

So the two-fold purpose of the current study were to determined the prevalence rate of *C. burnetii* in aborted Ovine and Caprine fetuses in Iran and evaluation of nested and real-time PCR assays to detection of *C. burnetii* in abomasal contents of aborted fetuses.

MATERIALS AND METHODS

Samples: From January to May 2010, a total of 782 Ovine and 744 Caprine aborted fetuses were collected from 108 commercial Ovine and Caprine dairy herds of ten provinces of different parts of Iran (Table 1). These samples had only abomasal contents of aborted fetuses that were collected under sterile conditions and were immediately transported to the laboratory in a cooler with ice packs. All abomasal content samples were kept at -20°C until processing.

DNA extraction: *C. burnetii* DNA was extracted by 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 according to the method described by previous study (Sambrook and Russell, 2001).

Nested PCR assay: All oligonucleotide primers were obtained from a commercial source (Cinna Gen, Iran). The nested PCR assay used to screen for *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 μL containing 5 μL of DNA sample, 0.5 mm MgCl2, 0.2 mM (each) dNTPs, 1 μm primer OMP1, 1 μm primer OMP2 and 0.5 U/reaction of Taq DNA polymerase (Promega, Madison, WI). The PCR assay was performed at 94°C for 4 min and then for 30 cycles of 94°C for 1 min, 56°C for 1 min and 72°C 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 μL containing 2 μL of DNA sample, 0.5 mm $MgCl_2$, 0.2 mm (each) dNTPs, 0.8 2 μm primer OMP3, 0.8 lmprimer OMP4 and 0.5 U/reaction of Taq DNA polymerase (Promega, Madison, WI). The PCR assay was performed at 95°C for 4 min and then for 30 cycles of 94°C for 1 min, 57°C for 1 min and 72°C for 1 min.

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 assay: The primer set consisted of primers trans-f (5′-GGGTAAAACGGTGGAACA ACA-3′) and trans-r (5′-ACAACCCCCGAATCTCATTG-3′). The internal probe trans-p (5′-AACGATCGCGTATCTTTAACAGCGCTTG-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 abomasal content sample was tested using the commercial kit (targeting the repetitive transposon-like region of *Coxiella burnetii*), LSI Taqvet *Coxiella burnetii*_ (Laboratoire Service International, Lissieu, France) assay, according to the manufacturer's instructions. The negative control sample used was

DNase RNase free water. The external positive control used was a solution containing 10^5 *Coxiella 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 Rotor Gene 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 of nested and real-time PCR assays: Solutions of purified *C. burnetii* were prepared ranging from 1×106 to 1 microorganisms/100 μL. DNA was extracted by a genomic DNA purification kit (Invitrogen, Paisley, U.K.), according to the instruction manual. About 200 μL of the solution at microorganism concentration were used and the extracted DNA was dissolved in 100 μl of distilled water. About 3 μL of the DNA solution were used in the nested and real-time PCR assays. Results are shown as number of microorganisms in one PCR-tube (microorganisms/PCR-tube) and in 200 μL of sample (microorganisms/sample).

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 test analysis were performed and differences were considered significant at values of $P<0.05$.

RESULTS

In this study a total of 782 Ovine and 744 Caprine aborted fetuses from 108 Ovine and Caprine dairy herds of ten provinces of Iran, were tested by nested and real-time PCR for presence of *C. burnetii*.

Results indicated that 98 out of 782 aborted Ovine fetuses (12.53%) and 122 out of 744 aborted Caprine fetuses (16.39%) were positive for presence of *C. burnetii* by nested PCR (Table 1).

After Real-time PCR, from a total of 782 and 744 aborted Ovine and Caprine fetuses, *C. burnetii* gene were distinguished in 121 (15.47%) and 152 (20.43%)samples, respectively (Table 1).

Sensitivity was compared between the nested and real-time PCR assays in the detection of *C. burnetii* DNA. The nested PCR assay with two pairs of primers, OMP1 and 2 and OMP3 and 4 detected 63 microorganisms/PCR-tube, equivalent to 2x103 microorganisms/sample, whereas the real-time PCR assay with the trans-f (5′-GGGTAAAACGGTGGAACA ACA-3′) and trans-r (5′-ACAACCCCCGAATCTCATTG-3′) primers, detected 9 microorganisms/PCR-tube equivalent to 2×102 microorganisms/sample (data not shown), indicating that the real-time PCR assay was 7 times more sensitive than the nested PCR assay.

Table 1: Distribution of *C. burnetii* in ten provinces of Iran by using nested PCR and real-timePCR. This study showed that *C. burnetii* in Caprine, has a more severe effects than Ovine

	No. of samples		Nested PCR (%)		Real-Time PCR (%)	
Provinces	Ovine	Caprine	Ovine	Caprine	Ovine	Caprine
Isfahan	89	81	5.00	10.00	6.00	12.00
			-5.61	-12.34	-6.74	-14.81
Gorgan	57	52	7.00	14.00	10.00	27.00
			-12.28	-26.92	-17.54	-29.67
Khozestan	83	77	18.00	21.00	22.00	25.00
			-21.68	-27.27	-26.50	-32.46
Khorasan	78	70	2.00	11.00	3.00	13.00
			-2.56	-15.71	-3.84	-18.57
Sistan va balochestan	71	75	3.00	4.00	5.00	6.00
			-4.22	-5.33	-7.04	-8.00
Kerman	79	68	6.00	5.00	7.00	6.00
			-7.59	-7.35	-8.86	-8.82
Gilan	92	91	19.00	23.00	23.00	22.00
			-20.65	-25.27	-25.00	-42.30
Fars	103	96	14.00	7.00	16.00	9.00
			-13.59	-7.29	-15.53	-9.37
Kordestan	75	64	13.00	15.00	16.00	18.00
			-17.33	-23.43	-21.33	-28.12
Ilam	55	72	11.00	12.00	13.00	14.00
			-20.00	-16.66	-23.63	-19.44
Total	782	744	98.00	122.00	121.00	152.00
			-12.53	-16.39	-15.47	-20.43

Statistical analysis showed significant differences about P<0.01 between aborted Ovine and Caprine fetuses for presence of *C. burnetii* by both nested and real-time PCR assays and P<0.05 between nested PCR and real-time PCR for detection of *C. burnetii*. In the other hand, The Ct values which obtained from real-time PCR had significant differences about P<0.01 between aborted Ovine and Caprine fetuses.

So this study showed the higher accuracy and sensitivity of real-time PCR than nested PCR to detection of *C. burnetii*in aborted Ovine and Caprine fetuses. Therefore the prevalence rates of *C. burnetii*in aborted Ovine and Caprine fetuses are 12.78% and 19.08% respectively.

In this present study, Khozestan (26.5%) and Gilan (42.3%) provinces have the highest and Khorasan (3.84%) and Sistan va balochestan (8%) provinces have the lowest prevalence rates of *C. burnetii*in aborted Ovine and Caprine fetusesin Iran, respectively (Table 1). Our results indicated that Caprine is more sensitive than Ovine to *Coxiella burnetii*'s abortion (20.43% in Caprine versus 15.47% in Ovine).

Results showed the high prevalence rate of *C. burnetii*in animals of Iran.

DISCUSSION

In the majority of cases, *C. burnetii*'s abortion occurs at the end of gestation without specific clinical signs until abortion is imminent, as observed with brucellosis or chlamydiosis. Aborted fetuses appear normal but infected placentas exhibit intercotyledonary fibrous thickening and discolored exudates, which are not specific to Q fever. A severe inflammatory response is observed in the myometrium and the stroma adjacent to the placentomal area during gestation in goats.

Because *C. burnetii* may be shed by other routes such as milk, feces, urine, placenta, or birth fluids, testing animal based on only abomasal contents of aborted fetus samples can be lead to misclassify the status of the animal (Guatteo *et al*., 2006). In fact, the differences between the prevalence of *C. burnetii* in ovine and caprine abomasal contents of aborted fetus samples found in this study may be because of the different routes of shedding *C. burnetii* present in these animals. Ovine shed *C. burnetii* mainly in feces and vaginal mucus, whereas Caprine excrete *C. burnetii* in their vaginal discharges, feces and milk (Rodolakis *et al*., 2007). Furthermore, the infected animals may not persistently shed *C. burnetii*. Shedding of *C. burnetii* by infected animals occurs mainly during parturition and lactation. Therefore, detection of *C. burnetii* in abomasal contents of Ovine and Caprine aborted fetuses not depends on the sampling time. In addition to above, abomasal contents of aborted fetuses contain all of the vaginal, placental and even birth fluids and it is an advantage of abomasal content samples than urine, feces and even milk samples for detection of *C. burnetii*.

In addition to the route of infection, the inoculums size is affect the expression of *C. burnetii* infection.

The *C. burnetii*'sabortion rate can range from 3-80% of pregnant animals (Berri *et al*., 2000; 2002; Palmer *et al*., 1983; Zeman *et al*., 1989). High abortion rates are rarely observed, except in some caprine herds (Palmer *et al*., 1983). Often, the number of animals those abort in the flock may not be enough to alert the farmer and human clinical cases often reveal the infection of the flock (Berri *et al*., 2000). Ewes shed more and longer in vaginal discharges than goats and can shed bacteria at subsequent pregnancies (Berri *et al*., 2003).

The epidemiology of Q-fever in Iran is essentially unknown and to the authors' knowledge, the prevalence rate of *C. burnetii* in ruminant's aborted fetuses in Iran has never been reported but there are some studies that reported detection of *C. burnetii* in various clinical samples in Iran (Khalili *et al*., 2010; Khalili and Sakhaee, 2009).

The prevalence rate of *C. burnetii* in aborted Ovine in Iran (15.47%), is higher than northern Spain (9%) (Oporto *et al*., 2006) and Italy (10%) (Masala *et al*., 2004) but is lower than Netherlands (up to 80%) (Roest *et al*., 2011) and the prevalence rates of *C. burnetii* in aborted Caprine fetuses in Iran (20.43%) are higher than United Kingdom (25%) (Jones *et al*., 2010) and lower than Netherlands (up to 80%) (Roest *et al*., 2011) and Italy (21.5%) (Parisi *et al*., 2006).

This present study showed that nested PCR and real-time PCR assays can be used extensively as fast, safe and accurate diagnostic methods to detection of *C. burnetii* in aborted Ovine and Caprine fetuses but the real-time PCR assay is faster and more sensitive and accurate than nested PCR to detection of *C. burnetii*. Previous studies suggested the high sensitivity, specificity and accuracy of molecular methods such as PCR (Arricau-bouvery *et al*., 2006), nested PCR (Fretz *et al*., 2007), PCR-Enzyme linked immune sorbent assay (PCR-ELISA) (Muramatsu *et al*., 1997) and Real-Time PCR (Guatteo *et al*., 2007) to detection of *C. burnetii* in clinical specimens.

Since, PCR has been developed for the detection of *C. burnetii* for a wide variety of clinical samples such as serum (Zhang *et al*., 1998), blood (Kato *et al*., 1998), urine (Vaidya *et al*., 2008), feces (Berri *et al*., 2000), milk (Willems *et al*., 1994) vaginal tissue (Cairns *et al*., 2007) and semen (Milazzo *et al*., 2001).

The real-time PCR assay that use in this study, is more accurate, sensitive and faster than ELISA and conventional PCR to detection of *C. burnetii*. 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. This study showed that the nested PCR method for detecting *C. burnetii* is technically time-consuming and labor-intensive than real-time PCR assay.

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

The controls of the coxiellosis in cases with abortions are very difficult. Because the vaccine was not able to prevent neither abortions nor bacterial shedding neither in milk, vaginal secretions and nor in feces (Souriau *et al*., 2003). Only Antibiotic treatments with tetracyclines can be used to reduce the number of abortions and the quantities of *C. burnetii* shed at parturition (Behymer *et al*., 1977).

Although no extensive prevalence study was undertaken, the results of this study indicate that the prevalence rates of *C. burnetii*'s abortions are high in Ovine and Caprine in Iran.

CONCLUSION

To our knowledge, this study is the first prevalence survey of *C. burnetii* in aborted Ovine and Caprine fetuses of Iran by evaluation of nested PCR and real-time PCR assays. This present study suggested that real-time PCR is more sensitive and more accurate than nested PCR and can use as a safe, fast and thrust full diagnostic method for detection of *C. burnetii*'s in aborted fetuses.

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