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Prevalence and some risk factors associated with brucellosis and leptospirosis in aborted fetuses of ruminant species

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Abstract

In this study, a total of 91, 88, 82, 50 and 35 aborted bovine, ovine, caprine, buffalo and camel fetuses respectively were tested for the presence of *Brucella* spp. and *Leptospira* spp. From the total of 697 animal herds, 124 out of 220 bovine (56.36%), 102 out of 190 ovine (53.68%), 96 out of 165 caprine (58.18%), 48 out of 73 buffalo (65.75%) and finally 31 out 0f 49 camel (63.26%) dairy herds were infected by both *Brucella* spp. and *Leptospira* spp. In total 69 (19.94%) and 107 (30.92%) out of 346 aborted fetuses were positive for *Brucella* spp. and *Leptospira* spp. by culture methods. For the detection of *Brucella* and *Leptospira* species, the DNA was detected by multiplex PCR from 79 (22.83%) and 120 (34.68%) out of 346 aborted fetuses, respectively. Also, 32 (9.24%) of aborted fetuses were diagnosed positive for presences of both *Brucella* and *Leptospira* species by multiplex PCR. This result indicated that all of the 69 *Brucella* and 107 *Leptospira* culture-positive samples had also positive multiplex PCR and in addition to this the multiplex PCR detected *Brucella* spp in 10 samples and *Leptospira* spp. Therefore, our results showed the sensitivity and specificity of multiplex PCR assay (93 and 100% respectively). Our results indicated that the multiplex PCR assay was an accurate, sensitive, fast, safe and specific method for the detection of *Brucella* spp. and *Leptospira* spp. in aborted fetuses. Further, the results showed that caprine was the most susceptible and camel is the least susceptible species to the two infecting bacteria. In addition to above, our results showed that the most cases of abortion due to *Brucella* spp. and *Leptospira* spp. occurred in the spring. Furthermore, our results showed that abortion caused by *Brucella* spp. and *Leptospira* spp. occurred mostly in first partum followed by second, third, forth, fifth and the last partum.

Keywords: Brucellosis; Leptospirosis; aborted fetuses; seasonal patterns; various partums

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Introduction

Brucella is a facultative intracellular Gram-negative aerobic bacterium and *Leptospira* is a motile spirochaetal bacterium (Munir et al., 2010; Magalhães et al., 2010). *Brucella* and *Leptospira* are pathogenic bacteria that cause brucellosis and leptospirosis in many species of animals including cattle, sheep, goat, camel, buffalo, dog and horse around the world (Cutler et al., 2005; Cheema et al., 2007). Both of these bacteria cause abortion in infected live stocks. *Brucella* spp. are classified into 8 species including while *Leptospira* spp. are classified into 16 species (La-ard et al., 2008; Antiabong et al., 2009).

The importance of these infectious diseases causes not only economic losses in the animal production, but also pose a potential risk to human health (Heinemann et al., 2000; Ocholi et al., 2005).

There are many factors that can induce abortions and premature births in pregnant animals. Therefore, according to high economic losses of brucellosis and leptospirosis, accurate, safe and sensitive diagnostic

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methods play a vital role in the control and eradication of this disease in animals and humans. Today, there are many laboratory methods for diagnosis of leptospirosis and brucellosis in human and animals. *Brucella* and *Leptospira* can be serologically diagnosed, but many factors may cause false positive and negative results and even cross reactions with other factors (Bolin 2003). Direct methods based on bacteriological tests are usually used, but they are dangerous, time consuming and difficult for the operators (Çetinkaya et al., 1999; Richtzenhain et al., 2002). In these years, in order to facilitate these problems, molecular diagnosis based on polymerase chain reaction (PCR) has been successfully employed for the detection of *Leptospira* and *Brucella* in clinical samples (Baquero et al., 2010; Moussa et al., 2011).

Multiplex PCR (m-PCR) is a kind of PCR derived procedure where multiple targets DNA sequences can be detected in a single reaction (Richtzenhain et al., 2002), therefore by application of this assay, the detection of *Brucella* spp. and *Leptospira* spp. may be successfully obtained in a single run from abortion cases of animals. Entirely, the epidemiology of abortions due to *Brucella* and *Leptospira* is essentially unknown around the world and the purposes of this study was to detect and assess the prevalence of brucellosis and leptospirosis in aborted fetus of bovine, ovine, caprine, buffalo and camel in various seasons and partum in Iran.

The purpose of this study was to find the prevalence of *Brucella* and *Leptospira* in aborted fetuses of bovine and buffaloe with respect to some risk factors through conventional and molecular techniques.

Materials and Methods

Samples collection

Brucella abortus strain 1119-3 and *LEPTOSPIRA interrogans* serovar *pomona* were kindly supplied by Zowghi Esmaeil from the Razi Institue of Karaj (Iran). From January 2010 to January 2011 in various seasons of the year, a total of 91 (124 bovine), 88 (102 ovine), 82 (96 caprine), 50 (48 buffalo) and 35 (31 camel) from commercial dairy herds (from a total 697 dairy herds in this area of Iran) were collected. These samples were collected randomly from first, second, third, forth and fifth and last five partum. In this study in order to avoid bacterial contamination during the necropsies of fetuses in a farm, and between farms, 10 ml of abomasal contents of aborted fetuses were collected by 21G sterile needle in a free environment, away from animal and humans and in the opposite direction of the wind.

All clinical samples collected from various parts of Iran and were sent under refrigeration to the Biotechnology Research Centre of Islamic Azad University of Shahrekord. They were stored at -20 ^{o}C until DNA extraction.

Brucella culture method

Bacterial isolation trial from samples were made on blood agar base (Oxoid) supplemented with 5% defibrinated sheep erythrocytes and antibiotics (Vancomycin, Nalidixic acid, bacitracin, nystatin and cyclohexamide at the dose recommended in OIE manual (2000). Cultures were incubated for 10 days with and without 5% CO_2 at 37°C. Bacterial isolated were identified according to the conventional procedures (Quinn et al., 1994).

Leptospira culture method

Abomasal contents of aborted fetuses were inoculated into the transport medium, which consisted of 87 mg of KH_2PO_4 and 644 mg of Na_2PO_4/liter and 1% bovine serum albumin. A 1:10 dilution of inoculated transport medium was then added to the culture medium, PLM-5 (Centeon, Kankakee, Ill.) along with 0.167% BBL agar and 200 µg of 5-fluorouracil/ml. Samples were incubated at 27°C for at least 2 months. Once a week, 5 µl of culture was viewed under 40X dark-field microscope for the presence of leptospires. A compact zone of growth approximately 1 cm from the meniscus was visible after 1 week in all positive cultures. The presence of *Leptospira* spp. in this zone was confirmed by microscopy.

DNA extraction

From each animal, 10 ml of abomasal contents of aborted fetuses were collected by 21G sterile needle. DNA extraction was performed according to the method of Consuelo Vanegas et al. (2009). Purification of DNA was achieved using a genomic DNA purification kit (Fermentas, GmbH, Germany) and the total DNA was measured at 260 nm optical density according to the method described by Sambrook and Russell (Sambrook and Russell, 2001).

Multiplex PCR

For the multiplex PCR method used from the Moshkelani et al method (Moshkelani et al., 2011). The oligonucleotide primers specific for *Brucella* spp. and *Leptospira* spp. were used in this study: 5'-CTA TTA TCC GAT TGG TGG TCT G-3' and 5'- GGT AAA GCG TCG CCA GAA GG -3' for *Brucella* spp. and 5'-GCG CGT CTT AAA CAT GCA AG -3' and 5'- CTT AAC TGC TGC CTC CCG TAG -3' for *Leptospira* spp. that designed from 16S ribosomal RNA gene of *Leptospira* (Accession No. FJ812170) and 31 kDa cell surface protein gene for *Brucella* (Accession No. DQ229169).

The m-PCR assay was performed in a final volume of 25 µL mixture containing 50 mM KCl, 10 mmol Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, 0.5 µmol of each primer, 1.25 unit Taq polymerase (Cinnagen, Tehran, Iran) and 5µL of DNA template. The expected size of amplicons includes 243 bp for *Brucella* spp. and 307 bp for *Leptospira* spp., the m-PCR assay employed the novel primers of PCR assays.

Reactions were initiated at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min and a final elongation step at 72°C for 5 min, with a final hold at 4°C. Amplified samples were analyzed by electrophoresis (120 V/208 mA) in 1.5% agarose gel and stained by ethidium bromide. A molecular weight marker (100bp ladder Fermentas) was used as size standard.

Sensitivity and specificity of multiplex PCR assay

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

Results

In this study, a total of 91, 88, 82, 50 and 35 aborted bovine, ovine, caprine, buffalo and camel fetuses were tested for the presence of *Brucella* spp. and *Leptospira* spp. through culture and multiplex PCR. Our results showed that all of the herds infected with *Brucella* spp. and *Leptospira* spp. Therefore, from the total numbers of 697 animal in this region, 124 out of 220 bovine (56.36%), 102 out of 190 ovine (53.68%), 96 out of 165 caprine (58.18%), 48 out of 73 buffalo (65.75%) and finally 31 out 0f 49 camel (63.26%) dairy herds were infected by both *Brucella* spp. and *Leptospira* spp. (Table 1).

In total 69 (19.94%) and 107 (30.92%) out of 346 aborted fetuses were positive for *Brucella* spp. and *Leptospira* spp. by culture methods (Table 1).

Gel electrophoresis of amplicons confirmed that all primer pairs specifically amplified the desired PCR products. Each PCR product was obtained as a clear band at 307 bp and 243 bp, generated by *Leptospira* and *Brucella*, respectively. For the detection of *Brucella* and *Leptospira* species, the DNA was detected by multiplex PCR from 79 (22.83%) and 120 (34.68%) out of 346 animal aborted fetuses, respectively. Also, 32 (9.24%) of aborted fetuses were diagnosed positive for presences of both *Brucella* and *Leptospira* species by multiplex PCR (Table 2). This result indicated that all of the 69 *Brucella* and 107 *Leptospira* culture-positive samples had also positive multiplex PCR. Therefore, our results showed that the sensitivity and specificity of multiplex PCR assay was 93 and 100%, respectively.

Our results indicated that the multiplex PCR assay was an accurate, sensitive, fast, safe and specific method for the detection of *Brucella* spp. and *Leptospira* spp. in aborted fetuses. Results showed that caprine is the most susceptible and camel is the least susceptible species to the two infecting bacteria (Table 2). The prevalence of both *Brucella* spp. and *Leptospira* spp. in winter (December-February), spring (March-

Table 1: Isolation of *Brucella* spp. and *Leptospira* spp. from aborted bovine, ovine, caprine, buffalo and camel fetuses by application of culture methods.

species	No. herds in the study region	Number herds studied	Number samples per herd	Number of samples	*Brucella* positive culture (%)	*Leptospira* positive culture (%)
Bovine	220	124	20-10	91	16(17.58)	26(28.57)
Ovine	190	102	15-8	88	19(21.59)	29(32.95)
Caprine	165	96	12-6	82	24(29.26)	36(43.9)
Buffalo	73	48	10-6	50	6(12)	9(18)
Camel	49	31	8-4	35	4(11.42)	7(20)
Total	697	401	20-4	346	69(19.94)	107(30.92)

Values in parenthesis show percentage prevalence

Table 2: Prevalence of *Brucella* spp. and *Leptospira* in aborted fetus samples from bovine, ovine, caprine, buffalo and camel herds by multiplex PCR in Isfahan province, Iran

species	Number of herds in the study region	Number of herds studied	Number of samples	Number of *Brucella* positive samples (%)	Number of *Leptospira* positive samples (%)	Number of both bacteria (%)
Bovine	220	124	91	(20.87) 19	(31.86)29	(7.69)7
Ovine	190	102	88	(25) 22	(37.5)33	(10.22)9
Caprine	165	96	82	(32.92) 27	(48.78)40	(14.63)12
Buffalo	73	48	50	(14) 7	(20)10	(6)3
Camel	49	31	35	(11.42)4	(22.85)8	(2.85)1
Total	697	401	346	(22.83)79	(34.68)120	(9.24)32

Values in parenthesis show percentage prevalence

Table 3: Distribution of *Brucella* spp. and *Leptospira* spp. in aborted animal fetuses in various seasons of the year

Species	No. positive samples (%)		December-February (%)		March-May (%)		June-August (%)		September-November (%)	
	Brucella positive (%)	*Leptospira* positive (%)	*Brucella* positive (%)	*Leptospira* positive (%)	*Brucella* positive (%)	*Leptospira* positive (%)	*Brucella* positive (%)	*Leptospira* positive (%)	*Brucella* positive (%)	*Leptospira* positive (%)
Bovine	(20.87) 19	(31.86)29	2	2	12	20	3	5	2	2
Ovine	(25) 22	(37.5)33	1	3	18	25	2	3	1	2
Caprine	(32.92) 27	(48.78)40	2	3	20	33	4	2	1	2
Buffalo	(14) 7	(20)10	1	1	3	6	2	2	1	1
Camel	(11.42)4	(22.85)8	1	1	2	5	1	2	-	-
Total	(22.83)79	(34.68)120	7(8.86)	10(8.33)	55(69.62)	89(74.16)	12(15.18)	14(11.66)	5(6.32)	7(5.83)

Values in parenthesis show percentage prevalence

Table 4: Distribution of *Brucella* spp. and *Leptospira* spp. in various partums of bovine, ovine, caprine, buffalo and camel species

species	No. positive samples (%)		First Partum		Second Partum		Third Partum		Fourth Partum		Fifth and top five	
	Brucella positive (%)	Leptospira positive(%)	Brucella positive (%)	Leptospira positive (%)	Brucella positive (%)	Leptospira positive (%)	Brucella positive (%)	Leptospira positive (%)	Brucella positive (%)	Leptospira positive (%)	Brucella positive (%)	Leptospira positive (%)
Bovine	(20.87)19	(31.86)29	13	12	4	14	1	2	1	1	-	-
Ovine	(25)22	(37.5)33	17	16	4	16	1	1	-	-	-	-
Caprine	(32.92)27	(48.78)40	21	19	4	17	2	2	-	1	-	1
Buffalo	(14)7	(20)10	5	5	1	4	1	1	-	-	-	-
Camel	(11.42)4	(22.85)8	3	4	1	4	-	1	-	-	-	-
Total	(22.83)79	(34.68)120	59(74.68)	56(46.66)	14(17.72)	55(45.83)	5(6.94)	7(5.83)	1(1.26)	2(1.66)	-(0.0)	1(1.26)

Values in parenthesis show percentage prevalence

May), summer (Jun-August) and autumn (September-November) seasons of the year have been shown in Table 3. The most cases of abortion due to *Brucella* spp. and *Leptospira* spp. occurred in the spring. Furthermore, our results showed that abortion caused by *Brucella* spp. and *Leptospira* spp. occurred mostly in first partum followed by second, third, forth, fifth and the last partum (Table 4).

Discussion

Leptospirosis has been diagnosed in Iran for many years by Microscopic Agglutination Test (MAT) and culture methods and the first application of PCR for detection of *Leptospira* in Iran, goes back to 2008 (Bokaie et al., 2008).

The prevalence rate of brucellosis in Iran in sheep and goat was 340/10,000 and in cattle was 56/10,000 (Lilenbaum and Santos, 1996) but 10810 new cases were reported to the Ministry of Health, with the incidence rate of 16.43/100 000 in Turkey, a neighboring country (Ministry of Health of Turkey, 2006).

Livestock rearing and management system is very important for the dispersion of leptospirosis (Mantur et al., 2007) but the transmission of *Brucella* spp. infection and its prevalence in a region depends upon several factors like social customs, food habits, husbandry practices, methods of processing milk and milk products, socioeconomic status, environment hygiene and climatic conditions, (Mantur et al., 2007).

PCR is applied for detection of various microorganisms, including clinical bacteria and viruses. Sensitivity of PCR is so high, that other methods such as isolation and culture of organisms could not compete with this method anymore.

By simultaneously amplifying of more than one locus in the same reaction, multiplex PCR has been determined as a rapid, accurate, safe and convenient screening assay, with both clinical and research uses. Simultaneous detection of brucellosis and leptospirosis in abomasal contents of aborted animals has been demonstrated in this study by analyzing a single sample using multiplex PCR.

Since, PCR has been developed for the detection of *Brucella* spp. in a wide variety of clinical samples such as semen (Kim et al., 2006), blood (Navarro et al., 2002), milk (O'Leary et al., 2006) and aborted fetus (Buyukcangaz et al., 2011) while it has been developed for the detection of *Leptospira* spp. in urine (Bomfim et al., 2008), kidney (Fearnley et al., 2008), aborted fetus (Richtzenhain et al., 2002), water (Tansuphasiri et al., 2006), blood (Shekatkar et al., 2010) and in all of these studies, PCR has been introduced as an accurate and sensitive assay for detection of these pathogens.

The detection of *Brucella* spp. by PCR in aborted clinical samples was only evaluated in cattle and sheep (Fekete et al., 1992; Çetinkaya et al., 1999) and the last

use of PCR for the detection of *Leptospira* spp. in aborted fetus of buffalo goes back to 2007 (Marianelli et al., 2007) and after that study there is no significant report on the detection of *Leptospira* spp. from the aborted buffalo fetuses. In another study a total of 4992 sera collected from domestic animal (4348), wild animals (112) and human beings (532) were tested for the presence of *Leptospira* spp. Most of these animals were reported to have significant abortions and it was found that 2.8% of buffalo samples were positive for presence of *Leptospira* spp. (Srivastava and Kumar, 2003). Seroprevalence of brucellosis in aborted fetus of bovine, ovine and camel showed that *Brucella* infections contribute significantly to abortion in cattle and goats but not in camels (Megersa et al., 2011) and this was similar to the findings of our study. Studies indicated that the multivariable logistic regression model on both individual and herd levels revealed large herds and contact with small ruminants as risk factors for prevalence of brucellosis in camel (Al-Majali, et al., 2008). In total, in extensive management system the prevalence of brucellosis among various species of animal is low (Mohammed et al., 2011). Camels are not known to be primary host for any of *Brucella* organisms but they are susceptible to both *B. abortus* and *B. melitensis* (Musa and Shigidi, 2001; Teshome et al., 2003).

So far, Kenya (77.5%) (Namanda et al., 2009), Turkey (40.11%) (Otlu et al., 2007), Libya (31%) (Ahmed et al., 2010), Iran (this study, 14%) and Sudan (23.8%) (Musa et al., 2008), had a highest prevalence of brucellosis respectively in bovine, ovine, caprine, buffalo and camel species and Turkey (25.42%) (Gummussoy et al., 2009), India (60.4%) (Sratname et al., 1992), Poland (89.8%) (Czopowicz et al., 2011), India (54.4%) (Selvaraj et al., 2010) and Iran (this present study, 22.85%), had a highest prevalence of leptospirosis respectively in bovine, ovine, caprine, buffalo and camel species in the world.

To the author's knowledge, infection in animals can be seen in any ages but *Brucella* and *Leptospira* have a higher durability in the animals which are sexually mature. In addition, bacteria have a higher durability in cold and humid weather. Therefore, in Iran, in spring season due to high humidity and low relative temperature had the most abortions (Table 3). In our study, both *Brucella* spp. and *Leptospira* spp. had a higher incidences in ovine and caprine fetus and we may conclud that caprine and ovine are the main sources of these two pathogens in Iran. In addition, our results showed that the majority of abortions caused by brucellosis and leptospirosis occurred from March to May and to the author's knowledge this period of time has coincided with lambing in Iran. Therefore, probably the main cause of abortions in bovine, buffalo and camel herds in Iran was close contact with infected ovine and caprine in lambing period. To the author's knowledge, livestock grazing in contaminated pastures, consumption of contaminated water and contact with aborted fetuses and infected fetal discharge, are the main routes for distribution of brucellosis and leptospirosis in the environment.

The primer that was designed in this study was analyzed using Gene Runner software. Software analysis of our novel primers compared with only one previous report (Richtzenhain et al., 2002) showed that, these primers having better qualified for secondary structures such as hairpins, self dimer, cross dimer and internal loops. The results indicated that multiplex PCR could be used as sensitive and fast assay for successful detection of *Brucella* spp. and *Leptospira* spp.

To our knowledge, this study was the first report of direct detection of brucellosis and leptospirosis by application of multiplex PCR in aborted bovine, ovine, caprine, buffalo and camel fetuses in Iran. Unfortunately, with all the alarms in these two infectious diseases throughout the world it is not only still fully eradicated but also, it has a high prevalence in some areas like Iran. Control of brucellosis and leptospirosis requires vaccination of healthy animals and elimination of infected ones.

Conclusions

Our data revealed that (i) bovine, ovine, caprine, buffalo and camel species were sensitive to both *Brucella* and *Leptospira* abortive agents (ii) caprine is the most susceptible and camel (for *Brucella*) and buffalo (for *Leptospira*) are the least susceptible species to *Leptospira's* and *Brucella*'s abortion (iii) multiplex PCR is an accurate, safe, fast, sensitive and specific assay for simultaneous detection of *Brucella* spp. and *Leptospira* spp. not only in abomasal contents of aborted fetuses but also in all clinical samples (iv) abortion caused by *Brucella* and *Leptospira* species has a seasonal pattern and out results indicated that most abortions occured in the spring season (v) the first partum animals are the most susceptible and fifth and with increase in partum the susceptibility are reduced (vi) vaccination and test and slaughter policy especially in those countries which have diseases in endemic condition, are the best ways for control and even eradication of these two major diseases.

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