

Full Length Research Paper

Detection of *Brucella* spp. and *Leptospira* spp. by multiplex polymerase chain reaction (PCR) from aborted bovine, ovine and caprine fetuses in Iran

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Brucellosis and leptospirosis are important causes of livestock abortion, that can be serologically diagnosed. Generally, direct methods as bacteriological isolation are employed, but they are dangerous, taking up much time and not enough reliable. The polymerase chain reaction (PCR) has been reported to be successful for simultaneous detection of microorganism. The aim of this study was to use multiplex-PCR as an accurate, safe and rapid method to detect *Brucella* spp. and *Leptospira* spp. in aborted fetuses of bovine, ovine and caprine herds in Isfahan province (Iran). DNA was obtained directly from the stomach contents of aborted fetuses and PCR was performed by two pair novel primers. In total of the 276 specimens, 40 (14.4%) and 25 (9.0%) were identified positive for *Brucella* spp. and *Leptospira* spp., respectively. The convenience and the possibility of detection of both bacteria at a time, strongly support the use of this mPCR for routine diagnostics.

Key words: Multiplex polymerase chain reaction (PCR), *Brucella* spp., *Leptospira* spp., abortion.

INTRODUCTION

Brucella is a facultative intracellular gram-negative aerobic bacterium of the genus *Brucella* and *Leptospira* is a motile spirochetal bacterium of the genus *leptospira*. *Brucella* and *Leptospira* are pathogenic bacteria that cause brucellosis and leptospirosis in many species of mammals around the world. Leptospirosis and brucellosis are widely distributed zoonosis that affects domestic and wild animals. Both of these bacteria cause abortion in infected cows. *Leptospira* and *Brucella* organisms may be excreted from affected animals in urine in large numbers and this is a source of transmission to in-contact animals and the environment with man at risk particularly from direct contact and urine contaminated water sources. The importance of these infectin desaiase not only to the economic losses in the

animal production, but also to risks to human health (Heinemann et al., 2000; Ochoi et al., 2005).

Although, abortions and premature births as reproductive problems may be the only clinical signals in pregnant animals but there are many factors that can induced these clinical signs. Today there are many laboratory methods for diagnosis of leptospirosis and brucellosis in human and animals. *Brucella* and *Leptospira* can be serologically (Gall and Nielsen, 2004; Naigowit et al., 2000) diagnosed, but many factors may cause false positive and negative results and even cross reactions with other factors (Kittelberger et al., 1995; Bolin, 2003). Direct methods based on bacteriological tests are usually applied, but they are dangerous, time consuming and difficult for the operators (Çetinkay a et al., 1999; Richtzenhain et al., 2002). Molecular diagnosis base on polymerase chain reaction (PCR) has been successfully described for the detection of *Leptospira* and *Brucella* (Gravekamp et al., 1993).

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Multiplex PCR (m-PCR) is a kind of PCR derived

procedure where multiple targets DNA sequences can be detected in a single reaction (LeyLa et al., 2003; Salehi et al., 2006) therefore by application of this assay, easily can detect *Brucella* spp. and *Leptospira* spp. at the same time from animals with abortions. The purpose of this study was to describe novel primers for m-PCR assay for the detection of *Brucella* spp. and *Leptospira* spp. in aborted fetuses of bovine, ovine and caprine herds in Iran.

MATERIALS AND METHODS

Brucella abortus strain 1119-3 and *Leptospira interrogans* serovar *pomona* were kindly supplied from the Razi Institute of Karaj (Iran). A total of 140 bovine aborted fetuses were collected from 45 commercial dairy herds in spring and summer of 2010. From March to May 2010 a total of 110 ovine aborted fetuses and 26 caprine aborted fetuses were collected from 32 and 15 sheep and goat breeding farms, respectively. All clinical samples were collected in Isfahan province (Iran) and sent under refrigeration to the Biotechnology Research Centre of Islamic Azad University of Shahrekord. All samples had only abomasal contents of aborted fetuses. They were stored at -20°C until DNA extraction. DNA was extracted from abomasum contents of aborted fetuses by using DNA extraction and purification kit (Cinnagen, Tehran, Iran) according to manufacturer's instruction.

The oligonucleotide primers specific for *Brucella* spp. and *Leptospira* spp. were used in this study, were: 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 were designed from 16S ribosomal RNA gene of *Leptospira* (Accession No. FJ812170) and 31 kDa cell surface protein gene for *Brucella* (Accession No. DQ229169).

The mPCR assay was performed in a final volume of 25 µl mixture containing 50 mmol KCl, 10 mmol Tris-HCl (pH 8.3), 1.5 mmol MgCl₂, 0.2 mmol of each deoxynucleotide triphosphate, 0.5 µmol of each primer, 1.25 unit Taq polymerase (Cinnagen, Tehran, Iran) and 5 µL of DNA template. The expected size of amplicons 243 bp for *Brucella* spp. and 307 bp for *Leptospira* spp., the mPCR 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 finalelongation 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 with 100 bp increments (100 bp ladder fermentas) was used as size standard.

RESULTS AND DISCUSSION

Gel electrophoresis of amplicons confirmed that all primer pairs were specifically amplified by 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 (Figure 1). The presence of *Brucella* and *Leptospira* species DNA were detected by multiplex PCR from 32 (22.8%) and 20 (14.2%) out of 140 bovine aborted fetuses, respectively. Also, all 26 caprine aborted fetus samples from 15 goat breeding farms were negative for *Leptospira* and only one sample was positive for

Brucella. Out of 110 ovine aborted fetus samples 7 (6.3%) and 5 (4.5%) was positive for *Brucella* and *Leptospira*, respectively (Table 1).

Brucellosis and leptospirosis are a diseases of economic importance to any livestock enterprise as it induces abortion in infected animals (Leal-Klezevas et al., 1995; Masri et al., 1997). *Brucella* and *Leptospira* infections usually occur after entering the infected animals to herds. Bacteria from different ways including infected animal secretions and even ingestion of contaminated food can be entered and can cause bacteremia. Therefore bacteria are attacking the placenta and cause abortion. *Leptospira* spp. and many species of *Brucella* such as *B. melitensis*, *B. abortus* and *B. ovis*, are the main factors causing abortion in the last 2 months of pregnancy in Iran.

Leptospirosis has been diagnosed in Iran for many years by MAT and culture methods (Abdollahpour, 1990; Maghami and Hoshmand, 1977) and the first using of PCR for detection of *Leptospira* in Iran, goes back to 2007 (Safavieh and Aghaiypour, 2007). Also, the first report on the isolation of *B. abortus* in Iran as the cause of abortion in cattle dates back to 1944 (Delpy and kaveh, 1945). Since then, this organism has been isolated on numerous occasions from bovine fetuses and milk of cows (Zowghi et al., 1984) and now the prevalence rate of brucellosis in sheep and goat was 340/10,000 and in cattle was 56/10,000 (Bokaie et al., 2008).

Reports indicate that the high prevalence rate of leptospirosis in Iran including Tehran (24.6%) (Maghami, 1980), mashhad (24.24%) (Talebkhan et al., 1996), Shiraz (32%) (Firouzi and Vandyousefi, 2000), Karaj (46.8%) (Goli, 2002), Guilan (22%) (Asadpour, 2002), Ahvaz (53.73%) (Hajikolaei et al., 2005) and The earliest study on the prevalence of leptospirosis (*L. interrogans*) in Iran indicated a positive rate of 31% in cattle and 17% in sheep (Maghami, 1967) but probably Leptospirosis in Iran is more prevalence than what it seems but since it identified has need to accurate laboratory analysis.

In turkey, the antibodies against *B. abortus* were detected in serum samples of aborted dairy cattle as 68.1, 65.6, 58.9 and 55.2% by the Competitive Enzyme-Linked Immunosorbent Assay (C-ELISA), Complement Fixation Test (CFT), Rosebengal Plate Test (RBPT) and Serum Agglutination Test (SAT), respectively. The total of 66 (40.5%) of sera were positive for *Leptospira* antigen (Gen et al., 2005).

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. Therefore, this method is a suitable approach to identify microorganisms in acute infections (Levett, 2001) and in these years the PCR technique has increasingly been used as a supplementary method for *Brucella* and *Leptospira* diagnosis (Romero et al., 1995; Baquero et al., 2010). By simultaneously amplifying

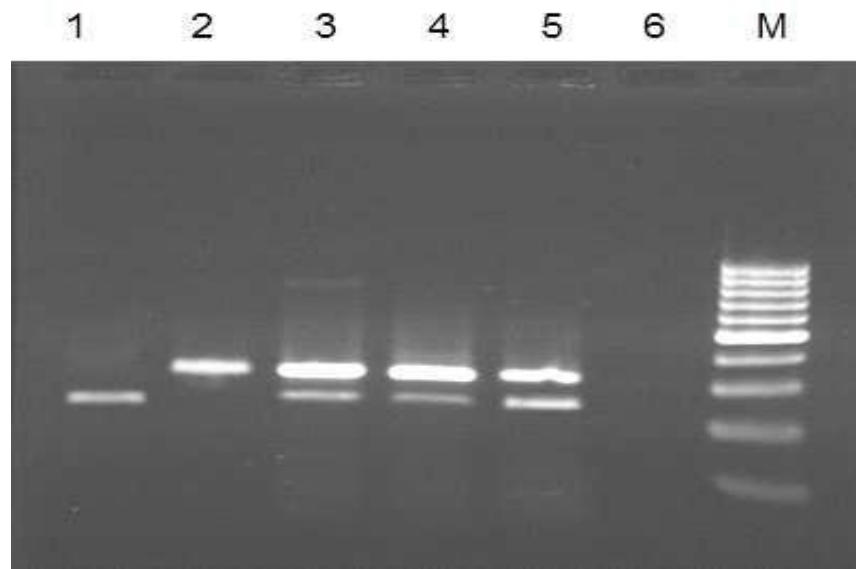


Figure 1. Multiplex PCR for the detection of *Brucella* spp. and *Leptospira* spp. Lanes 1 to 4: different samples from aborted fetuses; lanes 5: positive control; lane 6: negative control.

Table 1. Prevalence of *Brucella* spp. And *Leptospira* in aborted fetus samples from bovine, ovine and caprine herds in Isfahan province, Iran.

Herds	No. herds in the study region	No. herds studied	No. samples per herd	No. fetuses samples	No. (%) <i>Brucella</i> positive samples	No. (%) <i>Leptospira</i> positive samples	No. (%) both bacteria
Cow	120	45	3-9	140	32 (22.8)	20 (14.2)	5 (3.5)
Sheep	80	32	2-7	110	7 (6.3)	5 (4.5)	2 (1.8)
Goat	55	15	2-4	26	1 (3.8)	0 (0.0)	0 (0.0)
Total	255	92	-	276	40 (14.4)	25 (9.0)	7 (2.5)

more than one locus in the same reaction, mPCR has been identified as a rapid and convenient screening assay, with both clinical and research applications. Simultaneous detection of two major potential pathogenic bacteria in fetal stomach contents has been demonstrated in the present study by analyzing a single sample using mPCR.

The primers that designed in this study were analyzed using Gene Runner software. Software analysis of our novel primers compared with previous reports (Richtzenhain et al., 2002) shows that, these primers having better qualified of secondary structures such as hairpins, Self Dimer, Cross Dimer and internal loops. The results show that developed mPCR assay was able to successfully detect *Brucella* spp. and *Leptospira* spp.

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