

Detection of *Brucella* spp. and vaccine strains in bovine aborted fetuses by a multiplex PCR

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Abstract: The purpose of this study was to determine the seroprevalence of brucellosis among aborted cows and simultaneously to detect bacterial DNA in their aborted fetal tissues by means of PCR. In this descriptive study, peripheral blood samples were drawn from 76 cows aborted at the dairy farms of Tabriz (North-West of Iran) and their sera separated by centrifugation. The serum samples were analyzed by ELISA (Pourquire-ELISA Kit manufactured by France). Consequently, tissue samples were taken from the stomach (fluid), liver, kidney, spleen, lungs, heart and placenta of aborted fetuses and tested by PCR. Six out of 76 dams (7.8 percent) were seropositive to the *Brucella* spp., and six out of 76 aborted fetuses (7.8 percent) showed a positive reaction to the PCR test. Four out of six aborted fetuses (66 percent) showed a positive reaction against the *Brucella abortus* and the two remaining (34 percent) had a positive reaction to the vaccine strain, RB51. Statistical analysis did not show any significant difference between the two diagnostic methods (PCR and serological tests). However, PCR protocol is preferred to the serological tests due to its ability to differentiate among the *Brucella* strains. In conclusion, both serological and particularly PCR tests are recommended for diagnosis of *Brucella* strains in cows subjected to abortion and according to our PCR test results, vaccination with RB51 strain could be abortive in some pregnant cows.

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1. Introduction

Brucellosis of cattle, also known as contagious abortion and Bang's disease, is caused by infection from the bacterium *Brucella abortus*, which can also cause a disease of humans known as undulant fever (Radostitis et al., 2007). This disease causes abortion or premature calving of recently infected animals, most often between the fifth and eighth month of pregnancy. Infected cows frequently suffer from retained afterbirth, are difficult to get rebred, and are sometimes left sterile. The organism has an affinity for the reproductive tract and is spread from the vaginal discharge of an infected cow or from an aborted fetus. Infected breeding bulls can transmit the disease to cows at the time of service by infected semen (Noakes et al., 2009).

Because of the enormous losses the disease causes to the Iranian dairy and beef cattle industries (primarily due to abortion in the second half of gestation) correct and prompt diagnosis is important in controlling and eradicating the disease in this region. Efficient diagnosis requires a complete diagnostic protocol associated with submission of appropriate specimens and clinical history. Traditional diagnostic tools include serology, histopathology, bacterial isolation, and for certain agents direct examination or immunohistochemistry (Anderson, 2007). Direct methods based on bacteriological isolation are usually employed, but they are difficult, time consuming and dangerous (Richtzenhain, et al., 2002).

Since the early 1990s, PCR has been increasingly used as a diagnostic tool for etiologic diagnosis of abortion in cattle either as a complement or replacement of time consuming traditional diagnostic methods (Anderson, 2007). Several PCR and Rt-PCR (reverse transcription-polymerase chain reaction) protocols have been recently developed for identification of infectious agents in aborted bovine fetuses, including *Brucella abortus* (Leal-Klevezas et al., 2000; Cortez et al., 2001; Richtzenhain et al., 2002; Bricker et al., 2003). Considering the potential of PCR for etiological diagnosis of infectious bovine abortion, the objective of this study was to use PCR protocol as a tool for identification of *Brucella* spp. in tissues from aborted bovine fetuses and simultaneously ELISA as a complementary test for detection of *Brucella* antibodies in sera of dams subjected to abortion. This study included frozen tissues from aborted fetuses and sera from aborted dams.

2. Material and Methods

2.1 Samples

From May 2008 through August 2010, 76 blood and tissue samples were collected from aborted cows and their fetuses (n=76) at the dairy farms located in the Tabriz vicinity. Blood samples were centrifuged and sera harvested and kept at -20°C. Tissue samples were collected from several fetal organs including liver, kidney, lung, spleen, heart, stomach fluid and placenta, then separately

pulverized under liquid nitrogen and finally stored at -20°C until DNA extraction.

2.2 Enzyme- Linked Immunosorbent Assay (ELISA)

Using a commercial ELISA kit (Pourquier, France), sera were tested for the presence of antibodies to *Brucella abortus* according to manufacturer's instruction.

2.3 DNA extraction

DNA extraction from frozen tissues samples was performed using a commercial kit (Accuprep Genomic DNA Extraction Kit, Bioneer, S. Korea) following the manufacturer's instructions. Briefly, 100µL of thawed homogenates of fetal tissues were mixed with 600µL of Nuclei Lysis Solution and homogenized for 10 seconds. Samples were incubated at 65°C for 30min, followed by the addition of 17.5µL proteinase K (20mg mL⁻¹) and incubation at 60°C for three hours, vortexing every 30 min. Three microliters of RNase A (4mg mL⁻¹)

were added, the samples were mixed and incubated at 37°C for 30minutes. After cooling, 200µL of Protein Precipitation Solution were added, followed by vortexing and centrifugation at 13,000 g for 4minutes. The supernatant was transferred to a new microtube with 600µL of isopropanol, mixed, and centrifuged at 13,000 g for 3minutes. The supernatant was discarded and the pellet was washed with 600µL of 70% ethanol, followed by a final centrifugation at 13,000 g for 3min. Each pellet was dissolved in 100µL of DNA Rehydration Solution by incubating at 65°C for one hour.

DNA quality was assessed by spectrophotometry and PCR amplification of an internal control (prolactin gene). Samples that did not yield a prolactin amplicon nor had DNA concentration lower than 100ng µL⁻¹ as assessed by spectrophotometry were excluded from further analysis.

Table 1. Primer sequences for different *Brucella* species and strains.

No	Bacterial name	Primer sequence	PCR product molecular weight (bp)
1	<i>Brucella abortus</i> ,	5-AGCTGATCACATATGGGC-3 5-GACCATTACGTATCAACT-3	498
2	<i>Brucella ovis</i> ,	5- AGCTGATCACATATGGGC-3 5-CGGCTTCAGCCACCAACG-3	976
3	<i>Brucella melitensis</i>	5-ACGCCATCAATCAAGGGC-3 5-AATTCCCGTCCTTGGTGG-3	731
4	S19 strain	5-CTCCCGCTAAGAATT-3 5-CTCCATGTTAGCGGCGGT-3	178
5	RB51 Strain	5-AGCCGATCACTTAAG-3 5-GCCCGAAAGATATGCTTC-3	364
6	Rev1 Strain	5-TGGACCCCTTAGCCGTTGGACT-3 5-TCCACGGCAAGTCACGTTAACC-3	211

2.4 PCR

DNA samples were PCR tested for detection of *Brucella abortus*, *Brucella ovis*, *Brucella melitensis*, RB51 strain of *B. abortus*, S19 strain of *B. abortus* and Rev1 strain of *B. melitensis* by AMOS Multiplex PCR method (Bricker et al., 2003). PCR reactions were performed using 13µL of a commercial PCR mix (Accupower PCR preMix, Bioneer, S. Korea), 0.75µL of a 25pM solution of each primer (Table 1), and 1µL of DNA (100 to 500ng per reaction). Parameters used were initial denaturation at 95°C for five minutes, followed by

denaturation at 95°C for one minute, annealing at 55.5°C for one minute, extension at 72°C for one minute and a final extension at 72°C for seven minutes. The annealing temperatures and number of cycles for each agent are described, too. PCR products were resolved by electrophoresis in a 1.5 percent agarose gel stained with ethidium bromide. Positive controls included DNA from cultured organisms or infected tissues. Positive and non-template controls (in which the DNA template was replaced by PCR-grade water) were included with all reactions.

2.5 Statistical analysis

Frequencies of positive results were compared between PCR and ELISA tests by the Fisher's exact test, using SPSS software, version 16 (Graphpad Software, Inc., CA, USA).

3. Results

Six out of 76 dams (7.8 percent) were seropositive to the *Brucella* spp. and six out of 76 aborted fetuses (7.8 percent) showed positive reaction by the PCR tests (Table 2).

Table 2. Frequencies of abortions caused by *Brucella* Spp. detected by PCR (in fetal tissues) and ELISA tests (in dam's sera).

Test	Positive	Negative	Total
ELISA	6 (cows) (7.8%)	70 (cows) (92.2%)	76 (100%)
PCR	6 (fetuses) (7.8%)	70 (fetuses) (92.2%)	76 (100%)

Of six PCR positive samples, four fetuses (66 percent) showed positive reaction against the *Brucella abortus* and the two remaining (34 percent) against its vaccine strain, RB51 respectively (Fig.1 & Table 3). Statistical analysis did not show any significant difference between two diagnostic (PCR and ELISA) tests for overall detection of abortions caused by *Brucella* spp. However, only PCR protocol had the ability to differentiate *Brucella* strains from each other.

Table 3. Frequencies of abortions caused by *Brucella abortus* and RB51 strains detected by PCR in aborted fetal tissues.

Strain	Positive	Negative
<i>B. abortus</i>	4(66%)	2(34%)
RB51	2(34%)	4(66%)
Total	6(100%)	6(100%)

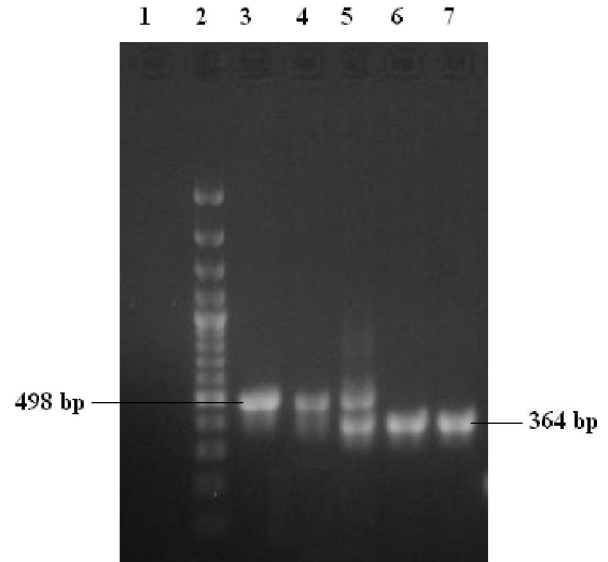


Fig. 1. Representative results of PCR amplification of genomic DNA of *B. abortus* and RB51 in fetal tissues: Lane 1: Non Template Control (NTC); Lane 2: molecular weight marker, Lane 3 & 4: samples from aborted fetuses; Lane 5: Multiplex positive control, upper lane (498 bp) for *B. abortus* and lower lane (364 bp) for RB51 strain; Lane 6 & 7: samples from aborted fetuses.

4. Discussions

Abortions have a highly negative impact on reproductive efficiency, resulting in significant economic losses for the cattle industry (Da Silva et al., 2009). The exact number of abortions due to infectious agents is not known, but in 90 percent of cases in which an etiologic diagnosis is achieved the cause is infectious (Nascimento et al., 2003). Brucellosis once was considered to be the most prevalent reproductive disease of cattle (Youngquist and Threlfall, 2007). Because of its major economic impact on animal health and the risk of human disease, most countries (including Iran) have attempted to provide the resources to eradicate the disease from the domestic animal population. Control programs have employed two principal methods: vaccination of young or mature animals, and the slaughter of infected and exposed animals, usually on the basis of a reaction a serological test (Radostitis et al., 2007). Serology is a standard method for the epidemiological surveillance of brucellosis (Leuenberger et al., 2007 and Köppel et al., 2007). However, cross-reactions between *Brucella* species and other Gram-negative bacteria are a major problem of the serological assays (Kittelberger et al., 1995, Muñoz et al., 2005). Molecular diagnostic techniques represent an important breakthrough in the diagnostic practice. A number of genus- or species-specific conventional PCR assays using

primers derived from different gene sequences from the Brucella genome, such as 16S rRNA (Herman, 1992), the 16S-23S intergenic spacer region (Rijpens et al., 1996) omp2 (Leal-Klevezas et al., 1995) and bcp31 (Baily et al., 1992) have been established. These assays were adapted for application to Brucella detection in different clinical specimens. In the majority of studies, PCR proved to be a good means to detect Brucella DNA from clinical specimens (Leal-Klevezas et al., 1995, Keid et al., 2007) while Romero and colleagues found that PCR had lower sensitivity compared to conventional detection methods (Romero et al., 1995). On the other hand Ilhan et al (2007) have emphasized the importance of using more than one type of diagnostic technique for the detection of animals positive for brucellosis, especially for epidemiological purposes (Ilhan et al., 2008). Based on the above-mentioned reasons and for more confidence, we decided to perform two different tests (PCR and ELISA) for diagnosis of abortions caused by Brucella spp. in the Tabriz dairy herds. Our results indicated that ELISA and PCR protocols have equal value for diagnosis of abortions caused by brucella spp. However, it seems that PCR protocol is more reliable than ELISA test because of its ability to determine different strains of Brucella spp. from each to other, particularly about the vaccine strains (RB51), where the positive animals must be slaughtered. In spite of the Iranian Veterinary Organization program for control and eradication of brucellosis, this disease is still a major threat for dairy herds of Tabriz (or Iran) and further investigation is required for eradication of this disease.

In conclusion, we recommend two different tests for etiological diagnosis of bovine abortions. One of these professional tests could be the PCR protocol, which is a very important tool for detection of bacterial strains, particularly in the cases of abortions caused by Brucella spp. Moreover, for the first time in Iran, our results indicated that the vaccine strain of Brucella abortus (RB51) is not fully safe and should be used cautiously in advanced pregnant animals.

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