

A serological and molecular (PCR) survey on abortions caused by *Brucella melitensis* Rev-1 vaccine strain in sheep herds of Tabriz-Iran

M. Saberi¹, H. Hamali^{2*}, R. Jafari Joozani², K. Nofouzi³ and GH. Noorsaadat⁴

- 1- Postgraduated student of Veterinary Faculty, The University of Tabriz, Iran
- 2- Department of Clinical Sciences, Veterinary Faculty, The University of Tabriz, Iran
- 3- Department of Pathobiology, Veterinary Faculty, The University of Tabriz, Iran
- 4- Tabriz Veterinary Network

Corresponding author: Hossein Hamali

ABSTRACT: Sheep and goats brucellosis is a zoonotic infection with important effects on both public and animal health and is widespread in many areas of the world, including Iran. The main purpose of this study was to determine of seroprevalence of brucellosis among the aborted ewes and in the same time, detection of bacterial DNA in the aborted fetal tissues by the PCR protocol. From October 2010 to March 2011, peripheral blood samples were taken from 100 ewes aborted in the farms of Tabriz (North-West of Iran) and their sera separated by centrifugation. Serum samples analyzed by ELISA (Pourquire-ELISA Kit manufactured by France). At the same time, tissue samples were taken from the abomasal fluid, liver, kidney, spleen, lung, heart and brain of the aborted fetuses and dam's placenta and tested by PCR. Twelve out of 100 dams (12%) were seropositive to the *Brucella spp.* and twelve out of 100 aborted fetuses (12%) showed positive reaction to the *Brucella melitensis* Rev-1 vaccine strain by the PCR. None of the aborted fetuses showed positive reaction to the *B. melitensis* standard strain (ATCC 23457). Statistical analysis did not show any significant difference between two diagnostic methods (PCR and serological tests). However, PCR protocol is preferred to the serological tests due to its ability to differentiate of the *Brucella* strains. In conclusion, both serological and particularly PCR tests are recommended for diagnosis of *Brucella* strains in the ewes subjected to abortion. According to our PCR test results, vaccination with *Brucella melitensis* Rev-1 vaccine strain could be abortive in pregnant ewes.

Keywords: Abortion, Brucellosis, Ewe, PCR, ELISA.

INTRODUCTION

Brucellosis in sheep and goats is a zoonotic infection with important effects on both public and animal health and is widespread in many areas of the world, particularly in some Mediterranean and Middle Eastern countries (Aras and Ates, 2011).

Brucellosis in human is mainly caused by *Brucella abortus* and *B. melitensis* biotype-1 which the later one is more prevalent and virulent than *B. abortus* (Jama'ayah et al., 2011).

In Iran, brucellosis was first recognized in 1949 and is now endemic throughout the country (Zowghi and Ebadi, 1982).

According to the Iran Veterinary Organization (IVO), *B. melitensis* biotype-1 is the main cause of small ruminant abortion in Iran. Unfortunately there is not a proper program (like the test and slaughter program in the cattle) for the eradication of small ruminant brucellosis and similar to the many other countries, the live attenuated strain *B. melitensis* Rev.1 ($0.5-3 \times 10^6$ per dose) is used for the prophylaxis of brucellosis in sheep and goats in Iran (Banai, 2002).

However, there are some evidences, demonstrating that attenuated *Brucella* vaccines strains used in animal's vaccination can represent a source of human and animal's brucellosis (Berkelman, 2003; Refai, 2002; Pishva and Salehi, 2008; Sharifi Yazdi et al., 2009; Beširović et al., 2011).

The Rev.1 vaccine is potentially virulent and apparently unstable, creating the requirement for innovation of new vaccines for controlling of *B. melitensis* (Banai, 2002). In addition, *Brucella spp.* may or may not provide cross-protection against infection by heterologous *Brucella* species. Also it is proved that vaccine can prevent abortion; but it probably can not provide complete protection against infection (Samartino et al., 2000).

Furthermore, this vaccine can induce abortion in pregnant animals and it is of great importance to demonstrate if this vaccine strain can transmit to unvaccinated sheep, goats and cattle (Pishva and Salehi, 2008).

It is not easy to distinguish between infections from vaccine or non-vaccine strains of *Brucella spp.* by the application of routine bacteriological and serological methods. The PCR protocol recently has been used as a method in detection of *Brucella spp.* and vaccine strains (Hamali and Jafari, 2011).

The main goal of this study was to determine of seroprevalence of brucellosis among the aborted ewes and in the same time, detection of bacterial DNA in the aborted fetal tissues by the PCR protocol.

MATERIALS AND METHODS

Samples

From October 2010 to March 2011, peripheral blood samples were drawn from 100 ewes aborted in the farms of Tabriz (North-West of Iran) and their sera separated by centrifugation and kept at -20°C. At the same time, tissue samples were taken from the abomasum(fluid), liver, kidney, spleen, lung and heart of aborted fetuses and dam's placenta. Then, separately pulverized under the liquid nitrogen and finally stored at -20°C until DNA extraction.

Enzyme Linked Immunosorbent Assay (ELISA)

Using a commercial ELISA kit (Pourquire-ELISA Kit manufactured by France), sera were tested for the presence of antibodies to *Brucella spp.* according to manufacturer's instruction.

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 with some modifications. 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 addition of 17.5µL proteinase K (20mg mL⁻¹) and incubation at 60°C for 3 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 30min. After cooling, 200µL of Protein Precipitation Solution were added, followed by vortexing and centrifugation at 13,000 *g* for 4min. The supernatant was transferred to a new microtube with 600µL of isopropanol, mixed, and centrifuged at 13,000 *g* for 3min. 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 1 hour. DNA quality was assessed by spectrophotometry and samples that had DNA concentration lower than 100ng µL⁻¹ were excluded from further analysis.

PCR

DNA samples were PCR tested for detection of *Brucella melitensis* and its vaccine strain Rev1 by AMOS Multiplex PCR method. 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 5min, followed by denaturation at 95°C for 1min, annealing at 55.5°C for 1min, extension at 72°C for 1min and a final extension at 72°C for 7min. Positive controls included DNA from culture of *Brucella melitensis* (ATCC 23457) or infected tissues. Positive and non template controls (in which DNA template was replaced by PCR-grade water) were included with all reactions. PCR products were resolved by electrophoresis in a 1.5% agarose gel stained with ethidium bromide.

Table 1. Primer sequences for *B.melitensis* and its vaccine strain: Rev-1

Bacterial name	Primer sequence	PCR product molecular weight (bp)
Forward <i>B. melitensis</i>	5'- CCGGATATGAATCTA ACC -3'	558
Reverse <i>B. melitensis</i>	5'- TGTACAAGGAACGCCA TCA-3'	
Forward Rev-1	5'-GGCATAACCTGCAGGAG CACT -3	211
Reverse Rev-1	5' -ATTACATCGGGCTCAAC TCG -3'	

RESULTS AND DISCUSSION

Results

Twelve out of 100 dams (12%) were seropositive to the *B.melitensis* and twelve out of 100 aborted fetuses (12%) showed positive reaction to the *B. melitensis* Rev-1 vaccine strain by the PCR (Table 2). None of the aborted fetuses showed positive reaction to the *B. melitensis* standard strain (Fig.1). Frequencies of positive results were compared between PCR and ELISA tests by the McNemar Test. Significant difference was not observed between two diagnostic methods ($P \geq 0.05$).

Table 2. The rate of abortions caused by *B.melitensis* vaccine strain Rev.1, in the sheep herds of Tabriz detected by the PCR and ELISA tests

Test	Positive	Negative	Total
ELISA	12(ewes)	88(ewes)	100
PCR	12(fetuses)	88(fetuses)	100

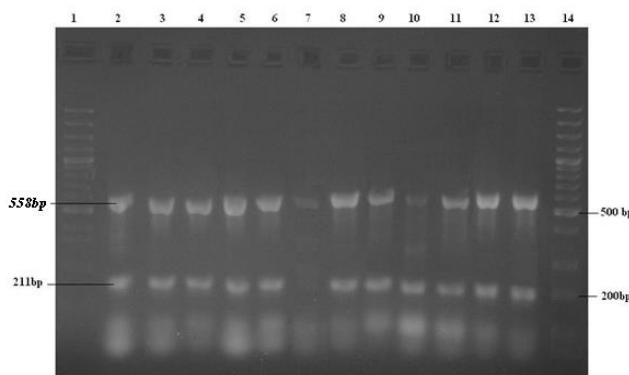


Figure 1. Representative results of PCR amplification of genomic DNA of *B. melitensis* vaccine strain Rev-1 in fetal tissues: Lane 1&14: 100 bp molecular weight marker (Bioneer, S. Korea) ; Lane 2: positive control (Rev-1 vaccine strain, Razi Institute, Karadj-Iran); Lanes 3- 6 &8- 13: positive samples from aborted ewes ; Lane 7: Positive control for *B. melitensis* (ATCC 23457)

Discussion

Abortions have a highly negative impact on reproductive efficiency, resulting in significant economic losses for the animal industry (Silva et al., 2009).

The exact proportion of abortions due to infectious agents is not known, but in 90% of cases in which an etiologic diagnosis is achieved the cause is infectious (Nascimento and Santos, 2003).

Brucellosis once was considered to be the most important reproductive disease of ruminants (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 of 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; its inability to distinguish between infections from vaccine or non-vaccine strains of *Brucella spp.* is a major problem of the serological assays (Kittelberger et al., 1995 and Muñoz et al., 2005).

On the other hand Ilhan et al., (2008) have emphasized on the importance of using more than one type of diagnostic technique for the detection of animals positive for brucellosis, especially with epidemiological purposes (Ilhan et al., 2008).

Based on 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 sheep herds. Our results indicated that ELISA and PCR protocols have the equal value for diagnosis of abortions caused by *brucella spp.* However it seems that PCR protocol is more reliable than ELISA test for distinguishing among the different strains of *Brucella spp.* including vaccine strains (Rev-1). This is very important in places where the positive animals must be slaughtered. On the other hand, despite the Iranian Veterinary Organization program for control and eradication of brucellosis, the wrong or untimely vaccination with the *B. melitensis* vaccine strain, may cause it as a major threat for sheep herds. Furthermore we exactly don't know that direct vaccination of pregnant ewes causes abortion or spread of *Brucella* vaccine strains from vaccinated animals to unvaccinated ewes lead to abortion. Further investigations are still required for clarifying the issue.

In conclusion, the results of the present study shows that combinational using of ELISA and PCR for etiological diagnosis of sheep abortions is a powerful method. However considering the ability of distinguishing among different strains of *Brucella spp.*, PCR has an advantage over ELISA. Moreover, for the second time in Iran, our results indicated that, the vaccine strain of *B. melitensis*, Rev-1; is not fully safe and could be lead to extensive abortions in pregnant ewes and should therefore be used with great care (Saeedzade et al., 2012).

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