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Original Article

Role of *Brucella abortus* Biovar 3 in the Outbreak of Abortion in a Dairy Cattle Herd Immunized with *Brucella abortus* Iriba Vaccine

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ABSTRACT

Bovine brucellosis is a widespread zoonosis caused by *Brucella abortus.* The disease is prevalent nationwide in Iran and is on an increasing trend among humans and livestock. The eradication of brucellosis is challenging and requires control policies at both national and regional levels. Regarding this, the aim of the current study was to evaluate if *Brucella* is implicated in an abortion outbreak that occurred in a dairy cattle herd, in Shahre Rey, Tehran province, Iran, after vaccination with *B. abortus* Iriba vaccine. The research context was a dairy cattle farm with 2,000 animals located in Shahre Rey. This farm was *Brucella*-free based on the results of two serological tests performed one month before vaccination. After the incidence of the first case of abortion following vaccination, serodiagnosis revealed a seropositive reaction in 30 non-pregnant cows and 19 pregnant cows that aborted later. Bacteriology and molecular typing facilitated the identification of 16 isolates of *B. abortus* biovar 3 from the aborted animals. None of the isolates were confirmed as *B. abortus* Iriba vaccine strain. The results confirmed that *B. abortus* biovar 3 was the most prevalent biovar in the cattle of Iran. The source and time of infection in the current study were not detected most likely due to the low biosecurity level in the farm (e.g., uncontrolled introduction of the agents via humans, infected animals, semen, and vectors). In endemic countries, the serodiagnosis of brucellosis alone is not sufficient and has to be accompanied by isolation and molecular diagnosis. In addition, it is important to evaluate the presence of *B. abortus* in bovine semen and vectors.

Keywords: Bovine brucellosis; Iriba vaccine; Abortion; *B. abortus* biovar 3

Rôle de *Brucella abortus Biovar 3* dans la Flambée des Avortements Survenue dans un Troupeau de Bovins Laitiers Immunisé avec le Vaccin *Brucella abortus Iriba*

Résumé: La brucellose bovine est une zoonose répandue causée par *Brucella abortus*. Cette maladie est présente dans toutes les provinces en Iran et la brucellose montre une incidence croissante, aussi bien parmi les hommes quele bétail. L'éradication de la brucellose est un défi et nécessite des politiques de contrôle aux niveaux national et régional. À cet effet, le but de cette étude était d'évaluer si cette infection était impliquée dans une flambée des avortement survenue dans un troupeau de bovins laitiers vacciné avec le vaccin *B. abortus Iriba* à Shahr-e-Rey dans la province de Téhéran, Iran. Cette recherche a été réalisée dans une ferme de bovins laitiers comprenant 2000 animaux située à Shahr-e-Rey. Cette ferme était exempte de *Brucella* spp. d'après les résultats de deux tests sérologiques effectués un mois avant la vaccination. Après la survenue du premier cas d'avortement après la vaccination des bovins, les résultats des tests sérologiques étaient positifs chez 30 vaches

non gestantes et 19 vaches gestantes ayant avortées ultérieurement Les analyses bactériologiques et le typage moléculaire ont permis l'identification de 16 isolats de *B. abortus biovar 3* provenant d'animaux ayant avortés. Cependant, aucun des isolats n'a été confirmé comme étant la souche vaccinale *B. abortus* Iriba. Les résultats ont confirmé que *B. abortus biovar 3* était le biovar le plus répandu chez les bovins d'Iran. Notre étude n'a pas permis de détecter la source et le moment de l'infection, probablement en raison du faible niveau de biosécurité dans la ferme (par exemple, introduction possible de nouveaux agents contaminés par l'homme ainsi que la présence éventuelle et incontrôlée de nouveaux animaux, spermes ou vecteurs infectés,). Dans les pays où la brucellose est autochtone, le sérodiagnostic de cette infection n'est pas, à lui seul suffisant et doit être accompagné d'un isolement et d'un diagnostic moléculaire. De plus, il est important d'évaluer la présence de *B. abortus* dans le sperme et parmi les insectes et parasites vecteurs des maladies bovines.

Mots-clés: Brucellose bovine; Vaccin Iriba; Avortement; *B. abortus* biovar 3

INTRODUCTION

Brucellosis is an endemic disease in most of developing countries that affects not only domesticated and wild animals but also humans. The disease is highly endemic among animals and humans in Iran. It is prevalent nationwide in this country and is on an increasing trend (Leylabadlo et al., 2015). As in most of endemic countries, the disease is affecting the people who have direct contact with animals. The highest rate of infection has been reported in the cities located in the west and north-west of the country (Pakzad et al., 2018). The eradication of brucellosis in Iran is a big challenge. In recent years, no well-designed control program has been implemented (Leylabadlo et al., 2015). In livestock, the disease induces severe economic losses as a result of infertility, abortion, and reduced milk production (McDermott and Arimi, 2002; Dadar et al., 2018). *Brucella melitensis* biovar 1 and *B. abortus* biovar 3 are the most frequently etiological agents among sheep and cattle in Iran, respectively (Pishva et al., 2015). It is important to note that the prevalence of brucellosis in humans is strongly related to the incidence of the disease in animals. To reduce the incidence of brucellosis in Iran, four strategies are applied, including passive surveillance, examination and removal of positive animals, certification of disease-free farms, and control of animal movements (Cárdenas et al., 2018). The surveillance of brucellosis in cattle is carried out by testing and slaughtering of positive animals in combination with vaccinating all negative non-pregnant cows and heifers older than 8 months and/or less than 3 months of gestation with *B. abortus* Iriba live attenuated vaccine (Esmaeili, 2015). However, some vaccine-related reproductive problems are still seen. With this background in mind, the current study was conducted to investigate if *Brucella* is implicated in the onset of abortion that occurred in a supposedly *Brucella*-free dairy cattle herd after vaccination with *B. abortus* Iriba vaccine.

MATERIAL AND METHODS

Evaluation of health status, serology, and vaccination of the herd. The present study was performed following an outbreak of abortion in a dairy cattle farm with 2,000 animals located in Shahre Rey, Tehran province, Iran. This farm was described as *Brucella*-free based on the results of two serological tests performed one month before vaccination. The screening of the farm was carried out under the supervision of Iranian veterinary services using the Rose Bengal test (RBT), serum agglutination test (SAT), wright test, and 2-mercaptoethanol (2ME) test as routine serological assays. Briefly, serum was separated from blood samples that were collected every 6 months for one year and transformed to a laboratory

for serology. All non-pregnant cows and heifers aged ≥ 8 months (n=300) and pregnant cows with the gestational age of ≤ 3 months (n=187) were vaccinated subcutaneously with 1-3.4×10^{10} CFU/dose of *B. abortus* Iriba vaccine (IRIBA vaccine, Iran). There was no injection site reaction after vaccination. The first case of abortion was reported 4 months post-vaccination. The screening of the farm by RBT, 2ME, and wright tests after the incidence of abortion revealed positive *Brucella* results in 19 pregnant and 30 non-pregnant cows. Abortions occurred later in all seropositive pregnant cases (n=19) at the 5th months of gestation. Other animals, such as dogs, cats, and wild birds, were in the vicinity of the infected animals. Cow fertilization in the farm was performed by artificial insemination using local sperms, which were not totally tested for possible *Brucella* infection. This study was approved by the Ethics Committee of Razi Vaccine and Serum Research Institute (RVSRI), Iran.

Bacteriological examination. Vaginal swabs and uterine discharges were collected from all cows that had abortions, as well as from the animals in contact with the infected ones. Fetal placenta and organs were collected from all aborted fetuses (n=20). All samples were collected under aseptic conditions, stored on ice, and examined via bacteriological tests under appropriate protection strategies with safety hoods at the Department of Brucellosis of the RVSRI. The primary isolation of *Brucella* species was performed by inoculating the samples on *Brucella* selective agar media supplemented with polymyxin B (2,500 IU), bacitracin (12,500 IU), nystatin (50,000 IU), cycloheximide (50.0 mg), nalidixic acid (2.5 mg), and vancomycin (10.0 mg) (Oxoid, UK), as well as inactivated 5% horse serum in *Brucella* agar (HiMedia, India). Then, they were incubated for 10 days at 37 °C with 10% CO_2. After 10 days of incubation, the typical colonies of *Brucella* species were subjected to further analysis for more identification and biotype analyses. Following Alton et al. (Alton et al., 1988), the identification and classical biotyping of *Brucella* isolates were performed based on colony morphology, biochemical reactions (oxidase, catalase, and urease), CO_2 dependence, H_2S production, agglutination with specific *Brucella* monospecific antisera A and M, growth in media containing thionin and basic fuchsin, and agglutination by acriflavine and phage lysis (Iz, Tb). The results were interpreted according to the OIE manual (http://www.oie.int/en/animal-health-in-the-world/animal-diseases/Brucellosis/2018).

Molecular identification. Genomic DNA was extracted from the heat-inactivated colonies. A loopful of bacterial biomasses was dissolved in 300 µl of molecular biology-grade water and then kept at 100°C for 15 min (Probert et al., 2004). The multiplex polymerase chain reaction (AMOS-PCR) was performed and confirmed the presence of *Brucella* species (Ewalt and Bricker, 2000). Multiplex Bruce-ladder PCR was performed for all strains as previously described (Lopez-Goñi et al., 2008). The DNA integrity was checked by 1% agarose gel. In addition, the concentration of DNA was evaluated at 260/280 nm by the Nanodrop Spectrophotometer (Wilmington, DE, USA) and stored at -20 °C until further analysis. The extracted DNA was subjected to IS*711*-based PCR for *Brucella* species. The PCR conditions consisted of five steps, including 95 °C for 5 min (step 1), 95 °C for 30 sec (step 2), 55 °C for 60 sec (step 3),72 °C for 3 min (step 4), and 72 °C for 10 min (step 5). Steps 2, 3, and 4 were repeated in 35 cycles (Ewalt and Bricker, 2000). In addition, the species-level molecular identification was performed using multiplex PCR (Bruce-ladder). This PCR was also composed of five steps, including 95 °C for 5 min (step 1), 95 °C for 30 sec (step 2), 56 °C for 90 sec (step 3), 72 °C for 3 min (step 4), and 72 °C for 10 min (step 5). Steps 2, 3, and 4 were repeated in 30 cycles (Lopez-Goñi et al., 2008). The amplified products were resolved by electrophoresis using 1 % agarose gel. All applied primers are described in Table 1.

RESULTS

The serological evaluation of the herd was performed after the incidence of the first case of abortion. The screening of the farm using RBT, SAT, and 2ME tests revealed a positive reaction in 50 cows. In addition to the cows with abortion, 30 cows from the non-pregnant vaccinated group showed a seropositive reaction; therefore, they were sent to a slaughterhouse for condemnation. Bacteriological examination facilitated the isolation of 13 and 3 *B. abortus* isolates from fetal organs and placenta, respectively. The isolated bacteria showed common phenotypic features typical for *Brucella* species. The isolated strains were Gram-negative and produced small honey-colored, translucent, and shiny colonies with a smooth surface. The isolates were characterized at the biovar level, and their identity was confirmed at the species level using the Bruce-ladder multiplex PCR and AMOS PCR, respectively. According to our results, all isolates were identified as *B. abortus* biovar 3. The results of AMOS PCR were indicative of the lack of *Brucella*-specific bands (Figure 1). Biovars 1, 2, and 4 can only result in 498-bp *B. abortus*-specific bands (Bricker and Halling, 1994). The use of the Bruce-ladder method led to the detection of *B. abortus* gene with the PCR products of

Table 1. Primer sets and expected amplicon sizes specific for different *Brucella* species

Strain amplicon	Primer set	Primer sequence (5-3')	DNA target	Size (bp)	References
B. abortus	IS711 AB	TGCCGATCACTTTCAAGGGCCTTCAT GACGAACGGAATTTTTCCAATCCC	IS711	498	(Ewalt and Bricker, 2000)
B. melitensis	IS711 BM	TGCCGATCACTTTCAAGGGCCTTCAT AAATCGCGTCCTTGCTGGTCTGA	IS711	731	(Ewalt and Bricker, 2000)
B.ovis	IS711 *B.ovis*	TGCCGATCACTTTCAAGGGCCTTCAT CGGGTTCTGGCACCATCGTCG	IS711	976	(Ewalt and Bricker, 2000)
B.suis	IS711 *B.suis*	TGCCGATCACTTTCAAGGGCCTTCAT GCGCGGTTTTCTGAAGGTTCAGG	IS711	285	(Ewalt and Bricker, 2000)
B. abortus *B. melitensis* *B. melitensis Rev.1*	BMEI0998f BMEI0997r	ATC CTA TTG CCC CGATAA GG GCT TCG CAT TTT CACTGT AGC	Glycosyltransferase, gene wboA	1,682	(López-Goñi et al., 2008)
B. abortus *B. melitensis* *B. melitensis Rev.1*	BMEI0535f BMEI0536r	GCG CAT TCT TCG GTTATG AA CGC AGG CGA AAA CAGCTA TAA	Immunodominant antigen, gene bp26	450	(López-Goñi et al., 2008)
Deletion of 25,061 bp in BMEII826–BMEII0850 in *B. abortus*	BMEII0843f BMEII0844r	TTT ACA CAG GCA ATCCAG CA GCG TCC AGT TGT TGTTGA TG	Outer membrane protein, gene omp31	1071	(Lopez-Goñi et al., 2008)
B. abortus *B. melitensis* *B. melitensis Rev.1*	BMEI1436f BMEI1435r	ACG CAG ACG ACC TTCGGTAT TTT ATC CAT CGC CCTGTCAC	Polysaccharide deacetylase	794	(López-Goñi et al., 2008)
B. abortus *B. melitensis* *B. melitensis Rev.1*	BMEII0428f BMEII0428r	GCC GCT ATT ATG TGGACT GG AAT GAC TTC ACG GTCGTT CG	Erythritol catabolism, gene eryC (Derythrulose-1-phosphate dehydrogenase)	587	(López-Goñi et al., 2008)
Deletion of 2,653 bp in BR0951–BR0955 in *B. melitensis* and *B. abortus*	BR0953f BR0953r	GGA ACA CTA CGC CACCTT GT GAT GGA GCA AAC GCTGAA G	ABC transporter binding protein	272	(Lopez-Goñi et al., 2008)
Point mutation in BMEI0752 in *B. melitensis* Rev.1	BMEI0752f BMEI0752r	CAG GCA AAC CCT CAG AAG C GAT GTG GTA ACG CAC ACC AA	Ribosomal protein S12, gene *rpsL*	218	(Lopez-Goñi et al., 2008)
B. abortus *B. melitensis* *B. melitensis Rev.1*	BMEII0987f BMEII0987r	CGC AGA CAG TGA CCATCA AA GTA TTC AGC CCC CGTTAC CT	Transcriptional regulator, CRP family	152	(López-Goñi et al., 2008)

1682, 794, 587, 450, and 152 bp (Table 1) in fetal organs and fetal placenta samples (Figure 2). Molecular assays confirmed all 16 strains as *B. abortus* biovar 3. None of the isolates in the farm were confirmed as *B. abortus* Iriba vaccine strain.

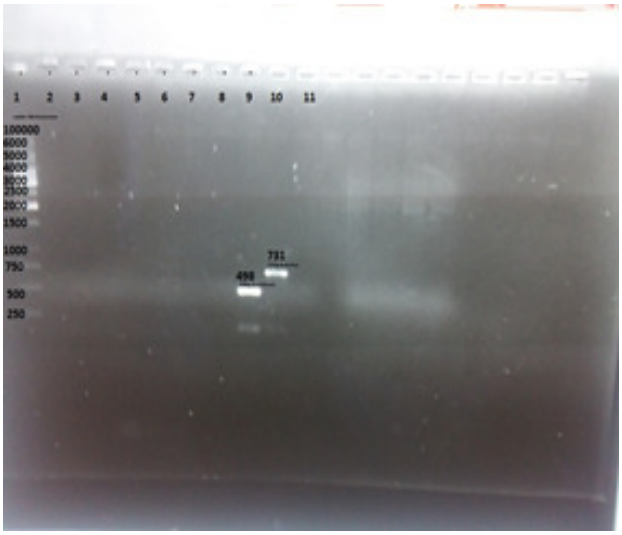

Figure 1. Agarose gel (1%) electrophoresis of polymerase chain reaction (PCR)-amplified products generated from DNA samples in AMOS PCR; lane 1) DNA size marker (1000-bp DNA ladder), lanes 2-8) no amplification for the DNA of the isolated bacteria, lane 9) 498-bp *Brucella abortus,* lane 10) 731-bp *B. melitensis* amplification products, and lane 11) negative control (Unspecific bonds in lanes 9 and 10 represent dimer primer.).

DISCUSSION

Brucella abortus strain RB51, also called Iriba in Iran, is a genetically stable and attenuated mutant of rough morphology that is currently used for the production of the official vaccine applied against bovine brucellosis in Iran (Schurig et al., 1991; Leylabadlo et al., 2015). The safety and immunogenicity of RB51 vaccine for cattle and pregnant cows have been confirmed in numerous studies (Singh et al., 2012; Barbosa et al., 2017). However, *B. melitensis* and *B. abortus* field strains have been reported in both goats and cows vaccinated with RB51 (Herrera et al., 2011; Arellano-Reynoso et al., 2013). Our results demonstrated the occurrence of abortion in a subgroup of Holstein dairy cattle herd after immunization with *B. abortus* Iriba vaccine in Shahre Rey. The farm had a *Brucella*-free status based on two serological tests performed one month before vaccination under the supervision of government veterinary services. *Brucella abortus* Iriba vaccine strain is a rough mutant of *B. abortus* that does not show the O-side chain of bacterium lipopolysaccharide in the surface, thereby producing no antibody reaction by serology (Singh et al., 2012). The results of the RBT, SAT, and 2ME tests demonstrated seropositive reactions in 20 pregnant and 30 non-pregnant cows.

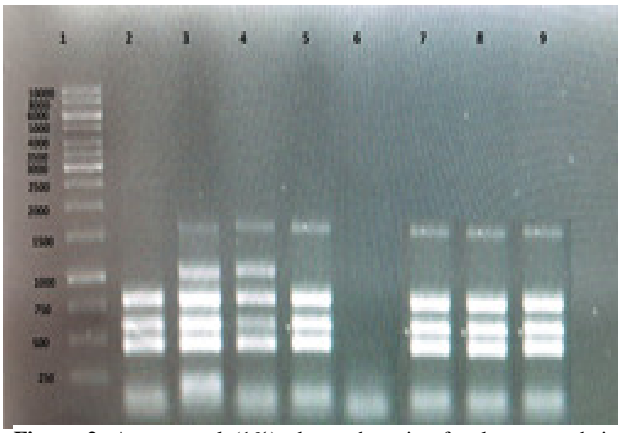

Figure 2. Agarose gel (1%) electrophoresis of polymerase chain reaction (PCR)-amplified products generated from DNA samples in Bruce-ladder PCR; lane 1) DNA size marker (1000-bp DNA ladder), lane 2) *Brucella abortus* Iriba, lane 3) *B. melitensis* Rev1, lane 4) *B. melitensis* 16 M, lane 5) *B. abortus* 544, lane 6) negative control, and lanes 7-9) *B. abortus* field strains.

According to bacteriology and molecular tests, abortions in the cultivated positive cases had been induced by *B. abortus* biovar 3. These findings are consistent with the results obtained in another study revealing the isolation of *B. abortus* from the uterine discharges of seronegative cows either vaccinated with RB51 (Wareth et al., 2016) or having no history of vaccination (El-Diasty et al., 2018). In addition, the DNA of *Brucella* species has been reported to be extracted from the semen of seronegative bulls (Junqueira Junior et al., 2013) and milk of seronegative cows (Islam et al., 2018; Sabrina et al., 2018). *Brucella*

abortus strains were also isolated from the vaginal exudate samples of a vaccinated cattle herd in Mexico using PCR and bacteriological tests (Arellano-Reynoso et al., 2013). Although Poester et al. (2000) reported no RB51 strain isolation from the milk or vaginal secretions of vaccinated animals, other studies performed on vaccinated cows revealed the isolation of this strain from the vaginal exudate and milk samples (Uzal et al., 2000; Leal-Hernandez et al., 2005). In another study, a *Brucella* species was also reported in the milk samples of serologically nonreactive buffaloes (Samaha et al., 2008). Likewise, the results of a study performed on 5,686 seronegative cows from Iran demonstrated 119 isolates of *B. abortus* in the milk samples (Zowghi et al., 1990). *Brucella melitensis* field strain was also isolated from the vaginal discharge of an RB51 vaccinated goat that had aborted in the third trimester and showed a seronegative reaction for brucellosis (Herrera et al., 2011). Furthermore, in a study performed in Damietta Governorate in Egypt, Wareth et al. reported seropositive reactions in the Holstein dairy cattle farms after vaccination with *B. abortus* RB51, using the SAT, complement fixation test, and RBT (Wareth et al., 2016). In the mentioned study, the farms were infected with a *Brucella* field strain that caused the majority of abortions. Culture-positive seronegativity is a serious problem resulting in the failure of control programs and further spread of infection to healthy herds. Based on the evidence, infected animals with a low antibody level or no circulating antibody could not be identified; therefore, they may have false-negative results (Bercovich et al., 1990) despite their infection with *Brucella* species. A low immunity condition in the animal during gestation can increase bacterial multiplication, thereby leading to the appearance of the clinical signs of brucellosis. Following vaccination, the clinical sign observed in our study was abortion in 20 pregnant cows; however, 30 non-pregnant cows were seropositive without any clinical manifestations. The presence of false-negative cases might be due to the low levels of bacteria that are insufficient to induce humoral immunological activity. Therefore, the serodiagnosis of brucellosis should be accompanied by molecular diagnosis before vaccination (Dadar et al., 2019). In the current study, the source of infection was not clear. However, other animals, such as dogs, cats, and wild birds, were seen in the vicinity of the infected animals and could also be contaminated with *Brucella* species. Furthermore, cow fertilization in the farm under investigation was performed by artificial insemination using local sperms, which were not tested for possible *Brucella* infection before use. It was not clear if the infection occurred before or after vaccination or whether the source of infection was infected animals as mentioned above and/or the uncontrolled introduction of the agent via farmers, infected semen, or other vectors. Serological tests prior to vaccination are not sufficient to diagnose brucellosis in endemic countries and have to be accompanied by isolation and molecular identification. Our results also suggested that the vaccination of cows with *B. abortus* Iriba vaccine could not be sufficient to eradicate and control brucellosis in cattle and should be accompanied by the implementation of continuous preventive programs to limit the new sources of infections.

Ethics

All procedures performed on animals were in accordance with the ethical standards established by the Ethics Committee of RVSRI, Agricultural Research, Education, and Extension Organization.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Authors' Contribution

Study concept and design: Alamian, S., Dadar, M.
Acquisition of data: Alamian, S., Dadar, M.

Analysis and interpretation of data: Dadar, M., Wareth, G.

Drafting of the manuscript: Alamian, S., Dadar, M., Wareth, G.

Critical revision of the manuscript for important intellectual content: Dadar, M.

Statistical analysis: Alamian, S., Dadar, M., Wareth, G.

Administrative, technical, and material support: Alamian, S., Dadar, M., Wareth, G.

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