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Original Article

The Control Program of Brucellosis by the Iranian Veterinary Organization in Industrial Dairy Cattle Farms

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Abstract

Brucellosis is a zoonotic infection in livestock that induces a major public health concern in developing countries, including Iran. Despite the efforts of the Iranian veterinary organization (IVO) to control brucellosis, it is still prevalent in domestic animals. In this regard, the present study aimed to evaluate the efficiency of the control strategy used by the IVO in infected herds on serological, cultural, and molecular methods. For this purpose, blood specimens were sampled from a total of 8750 vaccinated dairy cattle in two *Brucella*-infected farms. These farms were recognized as positive for *Brucella* by a screening program. Sera were evaluated by the Rose Bengal Plate Test and Wright test analysis. Positive dairy cattle were slaughtered under IVO supervision. The remaining cattle were evaluated every 3 weeks and positive animals were slaughtered. This procedure continued until the remaining animals revealed three successive negative responses in serological tests. Several lymph nodes and milk samples were collected from 164 seropositive cattle and subjected to bacterial isolation and confirmation by Bruceladder-polymerase chain reaction. *Brucella melitensis* biovar 1 and RB51 vaccine strains were recovered from milk and lymph node samples, respectively. Shedding of *B. melitensis* in the milk of vaccinated cows is a serious problem resulting in the further spread of brucellosis. The policy of "test and slaughter" performed on infected dairy cattle farms showed their usefulness for the control of brucellosis outbreaks. For the uncontrolled spread of brucellosis in Iran, effective control of bovine brucellosis required several serological surveillances to identify infected herds, eradication of the reservoirs, and vaccination of young heifers with RB51.

Keywords: *Brucella melitensis*, brucellosis, dairy cattle, RB51 vaccine

1. Introduction

Brucellosis is a significant threat to livestock and humans in many developing countries, It is particularly caused by *Brucella melitensis* and *Brucella abortus* in Iran. The bovine brucellosis in Iran is commonly caused by *B. abortus* and rarely by *B. melitensis*, as cattle are maintained in close contact with infected goats or sheep. Infection with *Brucella* spp. in cattle can persist for long periods and induce a serious challenge, despite decades of regulatory control programs worldwide.

Main reservoirs of the *Brucella* spp. are cattle, sheep, and goats, which shed the bacteria mainly through the milk, feces, semen, and urine of infected animals (1). Significant reproductive disorders have also been reported in sexually mature animals (2). The disease is mostly characterized by epididymitis, orchitis, and placentitis, but it is also manifested by late-term abortions, stillbirths, weak calves, and infertility (3). Although massive vaccination of cattle is potentially the most effective control approach, the administration of currently approved vaccines alone is

not sufficient for brucellosis elimination in any host species (4).

Humans easily become infected with *B. abortus* and *B. melitensis* due to the consumption of contaminated raw milk. Currently, S19 and RB51 are the live attenuated vaccines of *B. abortus* that are more widely used in the world to control bovine brucellosis. However, it is very important to note that there are no highly protective, safe, and effective vaccines for bovines. Calf-hood vaccination along with the One Health approach is highly demanded to control bovine brucellosis (5).

Bovine brucellosis has been reported in Iran since 1944 through the isolation of *B. abortus* from cattle (6). To prevent brucellosis outbreaks in livestock, a live attenuated RB51 vaccine, a rough mutant strain derived from the virulent strain of *B. abortus* 2308, has been licensed and used for many years in the cattle of Iran. This vaccine is a significantly useful approach for the control of bovine brucellosis through mass vaccine coverage for an adequately long period of time in combination with a proper test-and-slaughter program (7). However, without control measures for brucellosis in cattle, bacteria may circulate in the farms for several years.

The current field investigation aimed to monitor brucellosis outbreaks on two dairy cattle farms after the administration of full and reduced doses of the RB51 vaccine. Moreover, it aimed to evaluate the efficiency of the control strategies used by the Iranian veterinary organization (IVO) in the *Brucella*-infected dairy cattle farm on a serological, molecular, and cultural basis.

2. Materials and Methods

2.1. Herd History

Dairy cattle herds that had suffered from endemic abortion with 200 aborting cattle in a month were located in Kermanshah province of Iran (the western part of Iran). Furthermore, several stillborn calves were born until the end of the breeding season in April 2020. Further details about abortion during this period were not available. All cattle were kept under semi-intensive management and fed with concentrates and alfalfa hay during the daytime. The hay or silage from alfalfa was supplied from pastoral zones that had a history of small ruminant grazing. It should be noted that there was no contact between cattle and other domestic animals. However, animals were kept based on the overcrowded system in which pregnant and aborted animals as well as animals of different age groups and genders were housed together.

All calves were vaccinated with 1-3.4×10^{10} CFU of RB51 *B. abortus* vaccine for the first time (Razi vaccine and serum research institute, Karaj, Iran) in accordance with the recommendations of the manufacturer. Adult animals in herds of 1 and 2 were also annually revaccinated with a reduced dose of RB51 (1-3.4×10^{9} CFU). These farms were kept in quarantine by the authorities of IVO due to infection with *Brucella* spp.

2.2. Serological Analysis

The bovine sera of 8,750 specimens from two farms, namely farm one (n=4050) and farm two (n=4700), were separated by centrifugation at 3,000 rpm for 10 min. All sera were evaluated by Rose Bengal Plate Test (RBPT), and serum tube agglutination test (SAT) before vaccination with RB51 through the official brucellosis screening test of IVO. The antigens for RBPT and SAT were produced by the Razi Vaccine and Serum Research Institute (Karaj, Iran).

Sera specimens of cattle were considered to be positive at titers of 1:80 or greater (>1:80). Positive dairy cattle were slaughtered under IVO supervision. For detection of the remaining positive animals that incubated the disease, the animals were evaluated every 3 weeks based on the IVO serological tests along with the slaughtering of positive dairy cattle. This procedure continued until the achievement of three subsequent negative serological results.

2.3. Bacteriological Analysis

A sampling of milk (n=164) and different lymph node (n=164) specimens (retropharyngeal, prescapular, mediastinal, prefemoral, and supramammary) was performed through the seropositive cattle. Samples

from seropositive dairy cattle were subjected to bacterial cultures under safety hoods with appropriate protection. All individual samples of lymph node (n=164) and milk (n=164) were cultured on *Brucella* selective agar (containing Bacitracin (12,500 IU), Cycloheximide (50.0 mg), Nystatin (50,000 IU), Nalidixic acid (2.5mg), Vancomycin (10.0mg), and Polymyxin B (2,500 IU) (Oxoid, UK) in *Brucella* agar (HiMedia, Mumbai, India) with 5% inactivated horse serum) and kept for 14 days with 10% CO_2 in 37 ℃. It should be mentioned that a full identification analysis was performed on isolated bacteria (8).

2.4. Classical Biotyping of Bacteria

As previously described, a series of biotyping tests were performed for bacterial biotyping, including colonial morphology, agglutination by acriflavine, growth in the basic thionin and fuchsin media (contained 20-40 µg/ml), production of hydrogen sulfide gas (H2S), lysis by specific phages, dependence on 10% CO_2, and agglutination with polyclonal monospecific sera of anti-A and anti-M (9). Reference strains of *B. melitensis* 16M and *B. abortus* 544 were used as control isolates in classical biotyping.

The biochemical tests, colonial bacterial morphology, production of hydrogen sulfide gas, requirement for CO_2, growth on media with the inhibitory dyes fuchsin and thionin, agglutination with A and M and R polyclonal monospecific antisera, and phage typing by Izatnagar (Iz) and Tbilisi (Tb) according to the methods recommended by Alton et al. were used for bacteria identification (9).

2.5. DNA Extraction from *Brucella* Isolates and Molecular Typing

The DNA extraction from bacterial colonies was performed using the Exgene Cell SV kit (GeneAll, South Korea) according to the procedure recommended by the manufacturer. The DNA concentration was investigated by ND-1000 Nanodrop (Wilmington, USA) at 260/280 nm. The DNA integrity was analyzed with 1.5% agarose gel, and the DNA samples were stored at −20 ℃ for the next analysis.

2.6. Molecular Identification of Isolated Bacteria

Multiplex polymerase chain reaction (PCR) analyses (Bruce-ladder PCR) was performed for all bacterial DNA samples to identify bacterial isolates at the species level. The PCR conditions used in this study were as follows: initial denaturation for 5 min at 95 ℃ (step 1), second denaturation for 30 sec at 95 ℃ (step 2), annealing for 60 sec at 56 ℃ (step 3), extension for 3 min at 72 ℃ (step 4) and final extension for 10 min at 72 ℃ (step 5). Steps 2, 3, and 4 were repeated for 40 cycles (10). Moreover, the PCR reaction contained 2 µl of template DNA, 8-primer cocktail (0.5 µl for each primer), 7.9 µl of ddH2O, and 12.5 µl of Taq PCR Master Mix (0.1U Taq Polymerase/µl, 500 µM dNTP each, 20 mM Tris-HCl, 100 mM KCl, 3 mM $MgCl_2$). Finally, electrophoresis separation using 1.5% agarose gel was performed to evaluate the amplified PCR products.

3. Results

All sera of dairy cattle (n=8,750 specimens) were examined for the *Brucella* antibodies by RBPT and SAT (Table 1) in five steps. All sera of dairy cattle in farms one (n=4,050) and two (n=4,700) were seronegative before vaccination. Three weeks after the vaccination, a total of 97 (2.3%) sera specimens from farm one and 67 (1.42%) from farm two were serologically positive through the RBPT. Furthermore, 65 (1.6%) sera specimens from farm one and 54 (1.1%) from farm two showed seropositive reactions at titers of 1:80 or greater in SAT.

Serological examination of farms one (n=4,050) and two (n=4,700) using RBPT and SAT revealed a prevalence of one (0.02%) and two (0.04%) at the third examination, respectively (Table 1). Serial serological analysis was performed every 3 weeks for the identification of possible positive dairy cattle. The positive animals were slaughtered after

each test according to the IVO regulations. The first negative test results for the remaining dairy cattle were observed at the fourth test followed by the fifth negative serological examination, where the two herds were evaluated free from *Brucella* infection.

According to the culture results of 97 lymph nodes and 97 milk specimens of the seropositive dairy cattle in farm one, *Brucella* spp. were identified through the biotyping characterization in nine milk samples and nine lymph node samples. The bacterial isolates were recognized as *B. melitensis* biovar 1 in nine milk and eight lymph node samples. Furthermore, the RB51 vaccine strain also was isolated from one lymph node sample in farm one.

Bacterial cultures were also performed on 67 lymph nodes and 67 milk specimens of the seropositive dairy cattle in farm two. *Brucella* spp. were isolated and recognized from 10 milk and 5 lymph node samples. All bacterial isolates were recognized as *B. melitensis* biovar 1. The isolated bacteria showed single or small pairs of gram-negative coccobacilli with translucent honey round-like colonies. *Brucella melitensis* biovar 1 isolates represented growth on basic fuchsin and thionin dyes and were non-dependent on CO_2. They were all H2S-negative, and agglutination occurred with the monospecific anti-M serum but not the anti-A serum. They were lysed by Iz phages but showed non-lysis by Tb both in RTD and RTD10[4]. Only one isolate from the lymph node sample showed the properties of RB51 vaccine strains. Bruce-ladder PCR confirmed *Brucella* on genus and species level.

3.1. Polymerase Chain Reaction Assays

Bruce-ladder PCR was used to amplify the fragments of 1682, 794, 587, 450, 152, and 1,071 bp in size, showing the existence of *B. melitensis* at the species level. Furthermore, Bruce-ladder PCR of *B. abortus* RB51 vaccine strain amplified four fragments of 218, 587, 1071, and 1682 bp sizes and a specific additional 2,524-bp fragment. This confirms the previously reported results, which showed that *B. abortus* RB51 can be identified through the specific band of 2,524-bp (Figure 1) (10).

Table 1. Successive serological examinations of Brucella-infected dairy cattle farm

Location	Time of serological test	Number of sample examined	RBPT Positive N (%)	SAT Positive N (%)	Milk culture positive N (%)	lymphnode culture positive N (%)	Slaughtered cows	Isolate identified
Herd 1	1th (Before vaccination)	4050 cow	0	0	0	0	0	No isolate identified
Herd 2	1th (Before vaccination)	4700 cow	0	0	0	0	0	No isolate identified
Herd 1	2th (1 month after vaccination)	4050 cow	97 (2.3%)	65(1.6%)	9 (0.2%)	9 (0.2%)	97	*B. melitensis* bv1 RB51 vaccine strain
Herd 2	2th (1 month after vaccination)	4700 cow	67(1.4%)	54(1.1%)	10 (0.2%)	5(0.1%)	67	*B. melitensis* bv1
Herd 1	3th (2 month after vaccination)	3953 cow	1(0.02%)	1(0.02%)	0	0	1	No isolate identified
Herd 2	3th (2 month after vaccination)	4633 cow	2(0.04%)	2(0.04%)	0	0	2	No isolate identified
Herd 1	4th (3months after vaccination)	3952 cow	0	0	0	0	0	No isolate identified
Herd 2	4th (3months after vaccination)	4631 cow	0	0	0	0	0	No isolate identified
Herd 1	5th (6 months after vaccination)	3952 cow	0	0	0	0	0	No isolate identified
Herd 2	5th (6months after vaccination)	4631 cow	0	0	0	0	0	No isolate identified

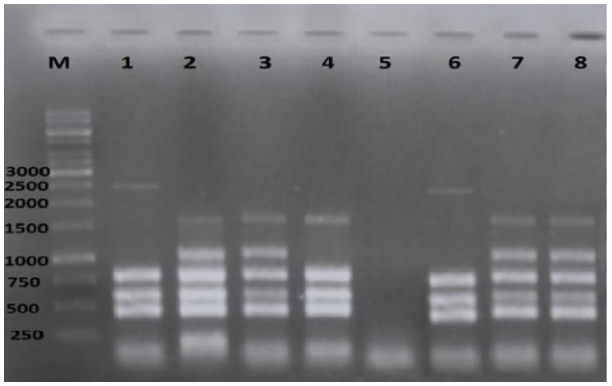

Figure 1. Bruce-ladder PCR products on agarose gel electrophoresis (1%). Lane M shows 1000bp DNA marker. Lane 1 shows *B. abortus* strain RB51; Lane 2 shows *B. melitensis* strain Rev1; Lane 3 shows *B. melitensis* 16M; Lane 4 shows *B. abortus* 544; Lane 5 negative control, Lane 6 represents the isolated RB51 vaccine from the lymph node. Lane 7 and 8 represents isolated *B. melitensis* from milk

4. Discussion

As there is no accepted cure for animal brucellosis, the only efficient approach for the control of brucellosis in dairy cattle is to test all the adult animals, slaughter the positive animals, and vaccinate all the calves. Vaccination plays a critical role in bovine brucellosis eradication/control programs and has been effectively used in different parts of the world. Control of possible reinfection of brucellosis is performed by the introduction of only brucellosis-free animals into the herd. Moreover, the eradication of brucellosis from male animals of the herd must be performed. The limitations and benefits of conventional brucellosis vaccines are outlined, and novel vaccination strategies are highly demanded to successfully protect animals against brucellosis (11).

The results showed that the seroprevalence rates of brucellosis in 8,750 cows using RBPT and SAT were 1.8% (n=164) and 1.3% (n=119) in the second examination. This prevalence may be due to a latent form of animal brucellosis with no or minimal amount of antibody production against *Brucella* and without infection of other heifers or cows in the herd. The identification of latent carriers cannot be performed through the RBPT and SAT and they may be identified serologically by evaluation of titers of both complement fixation test (CFT) or Coombs test before and after vaccination (12). Latent animals appear to be *Brucella*-free while maintaining the *Brucella* spp. within the herd (13).

It has been reported that about 20% of newborn calves from infected cattle could be persistently infected with *Brucella* spp. and may play an important role in the failure of the brucellosis control program (14). Furthermore, exposure of susceptible animals to vaginal and uterine discharges of infected animals is a critical risk factor for the transmission of brucellosis from infected to susceptible cases (15). Moreover, exposure of the susceptible dairy cattle to *Brucella* spp. through the addition of seropositive infected animals to herds without previous serological tests is considered another risk factor for the spread of brucellosis within the herds (16).

In this study, the slaughtering of infected animals could stop the spread of brucellosis in the herd. However, the serological evaluation of animals continued to detect seropositive animals in the consequent examinations for over 6 months. During this period, at least 8,583 dairy cattle were serologically negative for two consequent examinations with 3-week intervals. In these herds, the presence of *Brucella* spp. was analyzed based on milk and lymph node samples of infected animals over a period of 6 months. In this study, the clinical observations showed the shedding of *B. melitensis* from the milk of vaccinated cattle. Moreover, the results revealed the role of latent animals in herds as they do not show a detectable antibody response by the conventional serological tests used by IVO.

Infected milk from cattle is a potential contamination source of *Brucella* spp. that spreads the disease from animals to people (17). It has been proposed that there is a long interval between the development of clinical signs and *Brucella* infection (16). In a previous study, an apparently brucellosis-free heifer calf from a herd with *B. abortus* biovar 2 infection was sold to a

brucellosis-free herd. After nine years, *B.abortus* biovar 2 was isolated from the milk of this dairy cow and it elicited strongly positive serological titers (18).

Findings of the present study showed the outbreak of *B. melitensis* infection in dairy cattle farms. Efforts of the IVO to control *B. melitensis* in cattle by the use of the RB51 vaccine and regulated "test and slaughter policy" failed to reduce the prevalence of brucellosis or eliminate it as the prevalence of the disease has been reported in the last years (19). The difficulties of the implementation of the RB51 vaccine for the control of *B. melitensis* in cattle could be attributed to the lack of effective cross-species protection among *Brucella* species in cattle, although it has been proven through the investigation of recombinant vaccine of RB51 on mice models (20). Therefore, the slaughtering of all infected dairy cattle is one of the best applicable programs to control the outbreak of *B. melitensis* in the herd (21). However, a very strict management procedure is highly demanded for the possible eradication and control of *B. melitensis* in cattle.

Brucella melitensis, the most common cause of brucellosis in sheep and goats, is achieving growing importance in dairy cattle as an emergent zoonotic bacteria (21). It is also known as the most commonly reported bacteria in all the livestock of different countries. Infection with *B. melitensis* in cattle is a crucial issue for both veterinarians and farmers due to the lack of accurate knowledge about the persistence of infection in the herd and its transmission among cattle (22). Outbreaks of *B. melitensis* in dairy cattle in this study could be associated with the grazing of infected goat and sheep flocks in the surrounding area of farms.

Previously, *B. melitensis* infection in cattle has been documented in Iran (23). Prevalence rate of brucellosis in cattle was documented at 1.64% in Urmia City, Iran with failure of control approaches to eliminate the disease (24). Absence of a comprehensive surveillance policy to report the infected animals in large and small ruminants and the lack of border control for the movement of infected animals are known as the most important risk factors for brucellosis control in Iran.

Furthermore, the elimination of bovine brucellosis by test and slaughter policy looks impossible in developing countries, such as Iran, due to restricted budgets to pay farmers whose animals are eliminated and slaughtered during such screening tests. However, serological tests used in Iran by IVO for screening of brucellosis in dairy cattle farms are RBPT and SAT which could not detect latent forms of brucellosis and do not differentiate between historic and recent infections (12). In addition, most serological methods cannot differentiate between natural exposure to *Brucella* spp. and vaccine-induced exposure (such as *B. melitensis* Rev1 and *B. abortus* S19 vaccines). To improve the performance of serological tests, screening tests, including RBPT, can be improved by a confirmatory or complementary test, such as CFT or enzyme-linked immunosorbent assay (25).

Besides, the role of management control, such as eradication of infected animals, was highlighted in our study for control and elimination of bovine brucellosis. Achievement of brucellosis-free status in this study required 6 months which could be evaluated as a long period that spread the *Brucella* infection to other places, particularly under contaminated conditions, uncontrolled movement of animals, and mixed husbandry system of populations with different genders, ages, and pregnancy or abortion status. Therefore, effective control of bovine brucellosis in Iran highly demanded surveillance (screening and confirmatory or complementary tests) to identify infected herds, eradicate the reservoirs, and vaccinate young heifers.

Authors' Contribution

Study concept and design: M. D. and S. A.
Acquisition of data: A. B. and K. A.
Analysis and interpretation of data: M. D. and S. A.
Drafting of the manuscript: M. D.
Critical revision of the manuscript for important intellectual content: M. D. and S. A.
Statistical analysis: M. D.
Administrative, technical, and material support: A. B. and K. A.

Ethics

All animals in this study were treated according to the ethical standards for field studies approved by the Iranian veterinary organization in Tehran, Iran. All samples in this study were collected without causing any harm or stress to the animals based on the standard sample collection procedure. Informed consent was obtained from the dairy cattle farmers after that they were informed about the purpose of this investigation.

Conflict of Interest

The authors declare that they have no conflict of interest.

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