

First Report of Isolation of *Brucella melitensis*, Vaccine Strain Rev.1 as a Source of Cattle Infection in Iran

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Abstract

Brucellosis is a worldwide zoonosis causing reproductive failures in livestock. It is shown that although the vaccine can prevent abortion, it does not provide complete protection against infection. So that vaccination of ewes with Rev.1 biotype can be a source of cattle and even human Brucellosis. The aim of this study was to evaluate possibility of *Brucella* cross-infection in cattle from *Brucella* vaccine Rev.1 biotype in Iran. After *Brucella* vaccination in cattle and ewes in tradition farms in which cattle and sheep are kept close together, 70 aborted cattle fetus samples were collected. The species of *Brucella* were characterized and the type of *Brucella* was studied, by PCR-RFLP method of PstI restriction digest of part of *Omp2a* gene. Of the 70 studied samples, 50 (about 70%) were infected with *Brucella Spp*. Banding pattern of PCR-RFLP of *omp2a* gene demonstrated that in 2 of the samples (4%) the source of infection was Rev.1 vaccine. No sample was infected with Rb51 and S19 vaccine strains, which are specific for cattle vaccination. Our results demonstrated that Rev.1 vaccine biotype can cause *Brucella* infection in cattle. Therefore in places that cattle and sheep are kept close together, ewe vaccination (Rev.1) can be a source of *Brucella* abortion in cattle. This study has highlighted some of the potential hazards associated with the use of the Rev.1 vaccine in national control programs.

Keywords: PCR-RFLP; *B. melitensis* vaccine Rev.1; *B. abortus*

Introduction

Brucellosis is a worldwide zoonosis causing reproductive failures in livestock and a severe multi-organ disease in humans [1]. In Iran, brucellosis was first recognized in 1949 [21] and is now endemic throughout the country. The annual reported incidence

of human brucellosis is 26551 cases in 2005. The long-term serological studies have indicated that 5% of sheep and 0.8% of cattle was infected with brucellosis. *B. melitensis* infection is endemic particularly in Mediterranean and Middle East regions, parts of Africa and Latin America [2]. It is also the main cause of sheep abortion in Iran. The live attenuated strain *B. melitensis*

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Rev.1 is considered the best vaccine available for the prophylaxis of brucellosis in sheep and goats [4].

According to the Iran Veterinary Medicine Network, in Isfahan province, yearly removals of about 400 cattle reactors had little impact on the control of brucellosis. In this province also approximately 3,800,000 sexually mature sheep and goats are vaccinated with *B. melitensis* strain Rev. 1 (subcutaneous Lot) each year. Also approximately 450,000 dairy cows are vaccinated with *Brucella abortus* strain 19 and Rb51. *B. abortus* Rb51 vaccine is widely used to immunize the cattle [6,19], because it induces antibodies that do not react on standard serologic tests for brucellosis allowing differentiation between vaccination and infection [16, 19]. Economic losses due to brucellosis in livestock are considerable in an agrarian country like Iran and therefore vaccination is of great importance. But there are some evidences, demonstrating that there is real possibility that attenuated *Brucella* vaccines strains used in animal's vaccination can represent a source of human and animal's brucellosis [3,18]. *B. melitensis* biotype-1 in sheep, goats' cattle and man are the predominant infective biotype. *B. melitensis* biotype-1 is the most commonly isolated species and *B. abortus* biotype2 and, biotype3 are also rarely isolated from cattle and sheep [18]. Most human cases are caused by *B. melitensis*, particularly biotype 1. Both sporadic cases and epidemics occur in humans, but often the disease or infection is either unrecognized, or, if diagnosed, not reported to the public health authorities. Also brucellosis in camels has been reported in Iran [21].

The Rev.1 vaccine is quite virulent and apparently unstable, creating the need for improved vaccines for *B. melitensis* [4]. In addition, *Brucella* spp. may or may not provide cross-protection against infection by heterologous *Brucella* species, hampering the acceleration of vaccine development. It is shown that vaccine can prevent abortion; but it probably can not provide complete protection against infection [4, 19]. However, this vaccine can induce abortion in pregnant animals and also can represent a source of occupational risk for human brucellosis [3, 20].

In places with traditional farming, in which cattle and sheep are kept close together it is possible that ewe vaccination with *B. melitensis* Rev.1 vaccine to be a source of cattle infection. Therefore it is of great importance to demonstrate if this vaccine strain can cause infection in cattle. It is not easy to distinguish between infection from vaccine or non-vaccine strains of *Brucella* spp. by the application of routine bacteriological and serological methods. The PCR-RFLP analysis shows excellent typeability, reproducibility, stability, and epidemiological

concordance and has been used as a method in classification *Brucella* spp. [9,12]. The aim of this study was to evaluate possibility of *Brucella* cross-infection in cattle from *Brucella* vaccine Rev.1 strain.

Materials and Methods

This study was performed on traditional farms of Isfahan, in which both cattle and sheep are kept close together. Previously it was observed that rate of abortion, after *Brucella* vaccination, is relatively high in these farms. The total number of cattle in these farms was 540, of which 70 aborted cattle fetus samples were collected and, in less than 24 h after abortion, by the application of bacteriological methods the infectious agent causing abortion were characterized as follows:

Bacteriological Examinations

Samples from abomasums content and liver of the aborted fetuses were inoculated onto blood agar plates containing 7% defibrinated sheep blood with and without *Brucella* supplement (Oxoid, SR083A). The cultures were incubated at 37°C under an atmosphere with 10% CO₂. *Brucella* were identified by morphological, cultural and biochemical characteristics such as oxidase, urease, CO₂ requirement, H₂S production, growth in the presence of thionin and basic fuchsin (20 µg/ml). The strains were biotyped by agglutination with monospecific A and M antiserum [1].

DNA Preparation

DNA preparation for *Brucella* genomic DNA extraction, vaccines and *Brucella* sample strains were cultured for 24 h at 37°C on tryptic soy agar-yeast extract slopes and harvested, in 3 ml of sterile distilled water, by centrifugation at 2,000 × g for 10 min. The pellet was suspended in 567 µl of TE/sodium buffer (50 mM Tris, 50 mM EDTA, and 100 mM NaCl [pH 8.0]). Then, 30 µl of 10% (wt/vol) sodium dodecyl sulfate (SDS) solution and 3 µl of 2% (wt/vol) proteinase K solution were added, and the mixture was kept at 37°C for 1 h. The lysed cell suspension was extracted twice with phenol-chloroform, and nucleic acids were precipitated by gently mixing the aqueous phase with 2 volumes of cold ethanol. The precipitate was dissolved in 100 µl of TE (10 mM Tris, 1 mM EDTA [pH 8.0]). The amount of DNA was measured by electrophoresis of an aliquot of each sample through 0.8% agarose gels and was compared with standard DNA solutions [17]. The extracted DNA was then used for PCR amplification of part of *omp2a* gene.

PCR Amplification

The forward (F) and reverse (R) primers of *omp2a* gene were F 5'-GGCTATTCAAAATTCTGGCG-3' and R 5'-ATCGATTCTCACGCTTTCGT-3', respectively. PCR amplification was performed by the method of Mullis and Faloona [17]. A typical reaction mixture contained 50 mM KCl, 1.5 mM MgCl₂, 0.1% (wt/vol) Triton X-100, 0.2 mg of bovine serum albumin (fraction IV; per ml, and 10 mM Tris-HCl (pH 8.5). Each reaction mixture was supplemented with 100 mM each of the four deoxyribonucleotides, 100 ng of sample DNA, and each oligonucleotide primer. Reactions were initiated by adding 0.5 U of Taq polymerase [1, 12].

Following hot start treatment at 95°C for 3 min, PCR was performed with an Eppendorf thermocycler as follows: 40 cycles of PCR, with 1 cycle consisting of 1 min at 95°C for DNA denaturation, 2 min at 58°C for DNA annealing, and 3 min at 70°C for polymerase-mediated primer extension. 38 cycles consisting of 1 min at 95°C and 2 min at 58°C and 3 min at 70°C. The last cycle included incubation of the samples at 95°C for 1 min and then for another 2 min at 58°C and 10 min at 70°C. Ten µl of the amplified product was analyzed by electrophoresis in 1.5% agarose gels in TEA buffer (20 mM Tris-acetate, 1 mM EDTA [pH 8.0]).

DNA Digestion

PstI restriction enzyme was used according to the manufacturer's instructions. The digested DNA was separated by electrophoresis on either 1.5% agarose gels (wt/vol in Tris-acetate buffer) or 10% polyacrylamide gels (wt/vol in Tris-borate buffer). DNA fragments were visualized by staining with ethidium bromide (0.5 µg/ml) [1].

Positive and Negative Controls

DNA was also extracted from 4 vaccines strains of S19 and 2 different types of Rb51 (product of USA and product of Spain) and Rev.1. The last one is for ewe while others are for cattle vaccination. These DNA were used in PCR reaction as positive control. In negative control tubes water was added instead of DNA sample.

Results

Of the 70 studied samples, serological and molecular results demonstrated that 50 (about 70%) were infected with *Brucella* spp. Lanes 2, 6 and 7 of Figure 1 demonstrate a 1100 bp PCR products from *omp2a* gene. PstI restriction digest of *omp2a* gene PCR product

demonstrated 2 bands of 550 and 200 bp for S19 and Rb 51 vaccines, which are produced from *B. abortus* for cattle vaccination (Lanes 1, 2, 3 and 5 in Figure 2). Lane 4 of Figure 2 demonstrates PstI restriction digest pattern of Rev.1 vaccine strain that contains 3 bands of 282, 238 and 200 bp. Interestingly Rev.1 vaccine is used for ewe vaccination.

Out of 50 studied *Brucella*, 2 samples (4%) demonstrated banding pattern similar to Rev.1 vaccine strain, which means these 2 cattle get the infection from vaccinated ewes. Some samples produced 2 bands of 238 and 200 bp that represents either *B. melitensis* (other than vaccine strain) or *B. abortus* (Figure 2 lanes 6, 7, 8 and 9).

The rest of the samples (96%) were infected with other *Brucella* including *B. melitensis* biotypes other than vaccine strain and *B. abortus*.

Discussion

A retrospective data demonstrated that the Rev.1 vaccine led to the adverse effects of strain persistence in the vaccinated animals and was occasionally spread horizontally. The fact that vaccination did not always protect the animals in the field and the several cases of secretion of the field strain in milk had proven the inefficacy of the whole vaccination program [3, 20]. Human infection with the vaccine strain Rev.1 in South Africa has been reported, following horizontal infection among sheep [5].

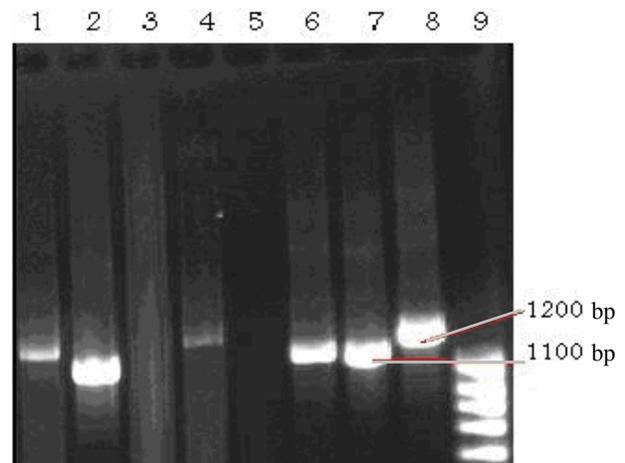


Figure 1. PCR product of *omp2a* gene amplification, *Brucella* spp. samples and vaccines strains. Lanes 2, 6, 7 *omp2a* lane 3 negative control, lane 5 did not work and lane 9 shows marker. Lanes 1, 4 and 8 PCR products of *omp2b* gene that was not used in this study.

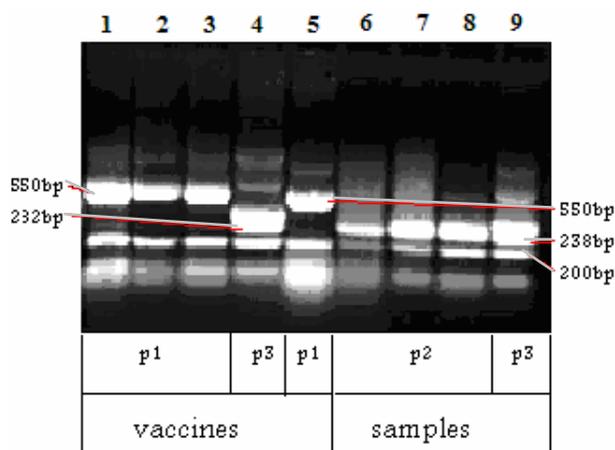


Figure 2. Restriction patterns of PCR-amplified *omp2a* gene cut by Pst1 enzyme. Pattern 1 (lanes 1, 2, 3, 5) consists of *Brucella abortus* vaccines strains Rb51 and S19. Pattern 2 (lane 4) consists of *Brucella melitensis* vaccine strain Rev.1 and (lanes 6, 7, 8, 9) pattern 2, 3 corresponding to *Brucella* spp. from the cattle fetuses samples.

Brucella is divided into six species and various biotypes differing in pathogenicity and host specificity [7]. There are some evidences showing that vaccination of ewes with Rev.1 strains can be a source of cattle Brucellosis. Vaccination against *Brucella* infections in animals is usually performed by administration of live attenuated smooth *B. abortus* strain S19, and *B. melitensis* strain Rev.1. They are proven effective vaccines against *B. abortus* in cattle and against *B. melitensis* and *B. ovis* in sheep and goats, respectively [4,6]. However, both vaccines have the main drawback of inducing O-polysaccharide-specific antibodies that interfere with serologic diagnosis of disease. In addition, they retain residual virulence, being a cause of abortion in pregnant animals and infection in humans. The potential for both exposure and for adverse consequences secondary to exposure to veterinary vaccines may be growing [20]. The *omp2* locus contains two gene copies (named *omp2a* and *omp2b*) coding for porin proteins and has been found particularly useful for molecular typing and identification of *Brucella* at the species, biotype, or strain level. *Omp2a* and *omp2b* genes encoding the group 2 porin proteins were identified in the late 1980s [13,15]. These genes, which are closely linked in the *Brucella* genome, and which share a great degree of homology (>85%), encode the 36 kDa porin proteins [11,14]. Analysis of PCR products of the *omp2a* and *omp2b* genes digested with 13 restriction enzymes indicated a greater diversity than the *omp25* gene among the six *Brucella* species and

within the *Brucella abortus*, *Brucella suis*, *B. melitensis* and *B. ovis* species. Greater polymorphism was also detected for the *omp2b* than for the *omp2a* gene [8, 10]. Curiously, the Pst1 digestion profile of the *omp2a* amplified fragments from the vaccine strain Rev.1 resembled *Brucella* spp. from the cattle fetuses samples. We assumed that because of the existing Pst1 site polymorphism between *B. melitensis* and *B. abortus*, the test would distinguish between the two species [5, 9]. According to the working hypothesis, DNA fragments obtained from *B. melitensis* strain biotype 1 should produce three fragments, an intact 282-bp fragment from the amplified *omp2a* gene that lacks the Pst1 site and two smaller fragments of 238 and 200 bp. In contrast, *B. abortus* vaccines strains (Rb.51, S.19) should produce only the two fragments from *omp2a* gene, a 550-bp fragment and a 200-bp fragment, respectively (Fig. 2). Results obtained by Cloeckert *et al.* [9]. Confirmed these data, showing that *B. melitensis* isolates were split between those with a single Pst1 site located in the *omp2b* gene and those with two Pst1 sites, one in *omp2a* and one in *omp2b*. Based on the diversity of both genes, distinction can be made by the use of PCR-restriction fragment length polymorphism (RFLP) between all *Brucella* species and some of their biotypes, except between *B. abortus* biotypes 3, 5, 6, 9, a number of strains of *B. melitensis* biotypes 1 and 3, *B. canis* strains and *B. suis* biotypes 3 and 4. [7]. Our findings show (Fig. 2) that the Pattern 1 acquired from PCR-RFLP consists of *B. abortus* biotype 1, 2, 4, R originated from the cattle *Brucella* vaccines (lane 1, 2, 3, 5). Pattern 2 acquired from PCR-RFLP consists of *B. melitensis* biotype 1, 2, 3 and R isolated from the *Brucella* spp. aborted fetus samples (Lane 6, 7, 8). Pattern 3 is observed both in *B. melitensis* Rev.1 vaccine strain (*B. melitensis* biotype 1, R) and, some of the samples obtained from cattle aborted fetus [7]. Therefore, some *B. melitensis* spp. causing abortions in the cattle are originated from *Brucella melitensis* Rev.1 strains (as determined by PCR-RFLP of the *omp2a* gene digested with Pst1) which we use for the vaccination of the bovine. This study has highlighted some of the potential hazards associated with use of the Rev.1 vaccine in national control programs.

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