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Detection of a *Brucella Melitensis* Rev1 Vaccine Strain and Rev1-Like Strains in Unvaccinated Small Ruminants and Aborted Fetuses

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Abstract

In this case study we report on the isolation of *Brucella melitensis* from two unvaccinated male animals (one ram and one buck) and from two aborted fetuses (lamb and calf). By applying both molecular and biochemical methods, investigating therefore the isolated strains at genus and species level they were classified in the case of buck as Rev1 vaccine strain while the rest three as Rev1-like since they exhibited some differences in their biochemical characteristics. It is worth mentioning that all the animals derived from intensively managed farm that had completely complied with the vaccination regiment.

Keywords: Brucellosis; Horizontal transmission; Rev1 Vaccine Strain; Blood-tissue Cultures; Molecular identification

Case History

The present study refers to the presence of *Brucella melitensis* in one buck identified as Rev1 vaccine strain and in one ram and two aborted fetuses identified as Rev1-like strains. The examined males were unvaccinated, originating from intensively managed farms that had completely complied with the vaccination regiment while the fetuses were aborted by vaccinated females.

The buck was three years old and originated from an intensive goat herd in Thessaly. The buck was bought one year earlier from an unvaccinated herd originated from a Greek island where the eradication program from brucellosis is applied (since 1992) The buck had been tested annually (1 year and 2 years of age) and found negative in serological tests. However, in August 2016, it was found positive in Rose Bengal Test (RBT) and Complement Fixation Test (CFT) performed by the authorized laboratories. In the same farm where all the replacements are being vaccinated at 3-7 months of age, last vaccination in replacements was performed in March 2016. The flock had been found negative at its annual test according to the national brucellosis surveillance program. In the flock there is one more buck which has been bought from the same herd as the positive buck but it was found negative. The incidence of abortions in the flock was stable ca. 2% the last years.

The ram was four years old, born and grew up in an intensive Chios sheep flock. Every year, as part of the annual serological testing in males according to the small ruminant surveillance plan against brucellosis, blood samples from the ram were tested and found negative. In October 2016 however, it was found positive in the RBT and CFT performed by the authorized laboratories. The last vaccination against brucellosis in flock level had been carried out at the replacement lambs in April 2016. In this flock, rams had been bought from two other flocks, both participating in the national vaccination program against brucellosis, and were found negative in serological tests performed at the flocks of origin. The abortions percentage in the flock was stable ca. 1%.

Blood samples were collected from the buck and the ram and were examined for the presence of the bacterium *B. melitensis* utilizing the lysis concentration method [1] in order to eliminate the possibility of infection from cross reactive bacteria [2-4]

Two aborted fetuses, a lamb and a calf, both originating from regions of Central Macedonia were also examined. The differential diagnosis of the abortions causes included among others, *B. melitensis* infection. Tissue samples were collected from the aborted fetuses and examined for the presence of *B. melitensis* after being found negative for *Salmonella* spp., *Listeria* spp. and *Escherichia coli* infections. Spleen, liver, stomach contents and placenta cotyledons samples were collected using sterile cotton swabs, during necropsy. Both the lamb and the calf were aborted at the last stage of gestation (four and eight months of gestation, respectively) by a vaccinated ewe and cow, respectively. The vaccination against brucellosis in both cases has been carried out using the vaccine strain B. *melitensis* Rev1. Since the detection of *Brucella* spp. wasn't the initial purpose of the analyses of the aborted fetuses no further data is available about the vaccinated ewe and cow and their flocks.

Ram's and buck's isolates were coded as B1 and B2 while the isolated strains from the aborted calf and lamb cultures, were coded as E1 and E2, respectively.

Discussion

The prevention of brucellosis in small ruminants is applied by national control programs including either obligatory vaccination in mainland Greece and the islands of Evoia, Lesvos, Limnos and Thasos or eradication of the disease (test and slaughter policy) in all the remaining islands [5]. Vaccination is carried out to all sheep and goats, usually between three and seven months of age during the conjunctival administration (5X 10^8-2X10^{10} CFU, Ministry of Agricultural Development and Food) of the attenuated live strain *B. melitensis* Rev1. Until 2016, the males were not vaccinated used as indicators of natural infection and in order to avoid the possibility of developing orchitis/epididymitis. In cattle, in certain endemic areas the vaccination has

been allowed in female cattle over four months old using the live strain *B. abortus* RB-51. Since 1999, the free-range cattle in the regions of Macedonia, Thessaly, Epirus and Central Greece, are exceptionally vaccinated with Rev1 vaccine strain, as infections by *B. melitensis* may occur due to the commonly practiced co-grazing with sheep and goats.

In our study, the isolated strains of the two animals and the two aborted fetuses (characterized as B1, B2, E1 and E2 as mentioned previously) were identified at genus level using molecular techniques. In all four strains the genus specific 520bp fragment of the insertion element IS711 was amplified (Figure 1A) and therefore, in accordance to the identification protocol of the OIE Reference Laboratory, all the isolates classified as *Brucella* genus. Furthermore, the amplification of the 400bp species specific fragment encoding omp-31 gene showed that all strains belonged to *B. melitensis* species (Figure 1B). Moreover, the comparison of the 16S rRNA sequences of the four isolated strains (GenBank accession numbers: B1- MK684236, B2- MK684240, E1- MK674615 and E2- MK681216) with those available at the GenBank, using the BLAST program (NCBI), confirmed that all belonged to *B. melitensis* species with a homology of up to 99% [6]. These findings were further supported by the results of the amplification of the *omp2* gene and the digestion with *PstI* that produced the digestion pattern of Rev1 vaccine strain in all the isolated strains (Figure 1C) where three DNA bands were present; the uncut *omp2a* fragment of *282bp* and the two fragments (238bp and 44bp) from the digested omp2b [7].

The analyses of the biochemical characteristics (Table 1) showed a 100% agreement with Rev1 vaccine strain in the case of the buck (B2). In the rest three isolated strains (B1, E1 and E2) the agreement with Rev1 was at the level of 67% to 83% and according to [7] they are classified as Rev1-like strains. Furthermore, the analyses of biochemical characteristics and their *PstI* digestion profiles of the *omp2* gene differentiate them from the other field strains isolated so far from various regions of Greece [8,9]. According to our findings, (resemblance of the biochemical characteristics and the similar digestion pattern of the *omp2* gene) it appears that the isolated strains from the ram and the aborted fetuses are possibly originating from Rev1 vaccine strain mutations as the ones studied in Israel by [7].

The detection of the Rev1 vaccine strain from the buck and the Rev1-like strain from the ram suggest a possible horizontal transmission of Rev1 either from the environment (e.g. possible contamination with abortion material, sharing of the same grazing pasture) or from the female sheep and goats to the unvaccinated males. Taking under consideration the fact that these animals were found positive after mating and about six months after the last vaccination in the flock, it seems likely that the vaccine strain is transmitted to males during mating with vaccinated females. In order to prove this possible horizontal transmission, a further careful designed field study needs to be performed.

Isolation of Rev1 vaccine strain in infected animals and/or aborted fetuses has been also reported in previous years elsewhere. In Iran it

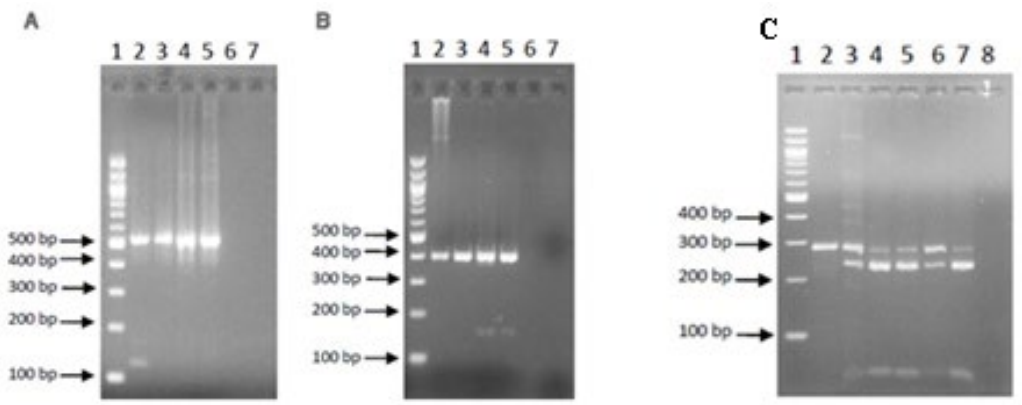

Figure 1: A: Results from PCR1. The genus specific 520 bp amplified fragment. Lane 1: 100 bp ladder, Lane 2: B1 strain, Lane 3: B2 Strain, Lane 4: E1 Strain, Lane 5: E2 Strain, Lane 6: -, Lane 7: negative control. B: Results from PCR2. The species specific 400 bp amplified fragment. Lane 1: 100 bp ladder, Lane 2: B1 strain, Lane 3: B2 Strain, Lane 4: E1 Strain, Lane 5: E2 Strain, Lane 6: -, Lane 7: negative control. C: Results from the PCR4 amplified fragments of the *omp2a/omp2b* gene digested with the PstI enzyme. Lane 1: 100 bp ladder, Lane 2: Rev1 strain PCR4 fragment undigested, Lanes 3-7 digested PCR4 products, Lane 3: Rev1 strain, Lane 4: E2 Strain, Lane 5: E1 Strain, Lane 6: B2 Strain, Lane 7: B1 Strain.
The electrophoresis was performed in 2% agarose gels.

Table 1: The biochemical characteristics of the four isolated strains. The analyses were performed also in the Rev1 vaccine strain and in *B. melitensis* 16M strain.

Sample	Fuchsin 20μg/ml	Thionin 20μg/ml	Streptomycin 2.5μg/ml	Penicilin 3μg/ml	Urease	H_2S
Buck	-	-	+	-	+	-
Ram	+	-	+	+	+	-
Aborted calf	+	-	+	+	+	-
Aborted lamb	-	-	+	+	+	-
Rev1	-	-	+	-	+	-
***B.melitensis* 16M**	+	+	-	+	+	-

was isolated from two aborted calves originating from unvaccinated cows that were kept in contact with vaccinated sheep [10]. In Egypt, ten seropositive ewes, of unknown vaccination status, were examined using bacteriological and molecular techniques and in two of them were identified Rev1 vaccine strains. Also, in Iran, forty six *Brucella* spp. strains were isolated from aborted lambs and goat kids. From them, five isolates were identified as *B. melitensis* Rev1 vaccine [11]. Similarly, in Turkey twenty four aborted lambs were examined and one *B. melitensis* Rev1 vaccine strain was isolated [12]. In both countries the animals were vaccinated with Rev1 vaccine strain. The horizontal transmission of Rev1 vaccine strain or its ability to survive in the vaccinated animals [13] has been suggested as the reason for these findings [7,14]. In Israel, Rev1 vaccine strain was isolated from the milk of two unvaccinated ewes indicating a milk-borne transmission of the vaccine strain [14]. The survival ability and the horizontal spread of the vaccine strain are of great significance for the public health. Human infections with the Rev1 vaccine strain in have also been reported in South Africa where the Rev1 vaccine strain has been transmitted horizontally from sheep to human. Two cases of human infections with Rev1 vaccine strain were recorded in Israel, one isolated from the owner of an intensive sheep flock and one from a 15-year-old girl [7].

This is the first time that the Rev1 vaccine strains and Rev1-like strains have been isolated from unvaccinated small ruminants (buck and ram) and aborted ruminants (lamb and calf fetus) in Greece.

Applied Methodology

Bacterial cultures were performed into Tryptic Soy Broth (TSB) supplemented with 5% horse serum, 7% sucrose and antibiotics (Polymyxin B = 5 IU, Bacitracin = 25 IU, Nalidixic acid=5 μg, Nystatin= 100 I.U., Vancomycin= 20 μg, Actidione= 100 μg). The cultures were incubated at 37 °C aerobic and in the presence of 10% CO_2. In total 6 subcultures were performed in accordance with World Organization for Animal Health (OIE - Reference Laboratory of Brucellosis in Italy) protocol and the duration of each culture was 2 months. Bacterial strains having the same morphology with *Brucella spp*. (from the aerobic cultures) were isolated from all the analyzed samples. The basic biochemical characteristics of *Brucella spp.* [culture in presence: of dyes basic fuchsin and thionin (20μg/ml), penicillin (3μg/ml) and streptomycin (2.5μg/ml), urease activity and production of H_2S] were used for the identification of the isolated strains. Ram's and buck's isolates were coded as B1 and B2, respectively, while the isolated strains from the aborted calf and lamb cultures, were coded as E1 and E2, respectively.

DNA extraction from the isolated strains was performed in 3 ml of a three days broth culture following the instructions of the PureLink Genomic DNA Mini kit (Life Technologies) for Gram negative bacteria. In total four different PCR reactions were performed; PCR1, PCR2, PCR3 and PCR4. The PCR1 was performed for the detection of *Brucella* genus (unpublished primers from the reference laboratory of OIE in Italy) resulted to a fragment of 520 bp of the insertion element IS711. The PCR2 was performed for the detection of *B. melitensis* species. Species' specific primers (VRI-F 5'-TGTTGACACCTTCTCGTGGA-3', VRI-R 5'-CAGGTTGAACGCAGACTTGA-3') were designed on the gene encoding for the outer membrane protein omp-31 gene in our laboratory. The PCR3 was performed for the amplification and sequencing analysis of an approximately 1500 bp fragment of the 16S rRNA gene [15]. The 16S rRNA gene was amplified by PCR using primers 8F and 1541R. The PCR products were sequenced directly by the dideoxynucleotide chain-termination method using a DNA sequencer ABI Prism 3100 and ABI Prism 3730XL according to the manufacturer's instructions. The PCR4 was performed for the amplification of a 282 bp fragment of the omp2 gene that was digested with the PstI [7].

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