Identification and Differentiation of *Brucella Melitensis* Rev.1 Vaccine and *B. Melitensis* Biovar 3 Field Isolates in Egypt by Serological and PCR-RFLP Techniques

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**Abstract:** Serum samples were obtained from 32 ewes from different localities in Menufia governorate of unknown history of vaccination or infection. These samples were subjected to serological examination for detection of specific brucella antibodies. Among these animals, 10 (31.25%), 8 (25.55%), 7 (21.88%) and 7 (21.88%) were positive for Standard tube agglutination test (SAT), Rose Bengal plate test (RBPT), Rivanol test (Riv T) and complement fixation test (CFT), respectively. Tissue samples were collected from seropositive animals. *Brucella melitensis* were detected in tissue samples collected from only 6 seropositive animals. Characterization of *B. melitensis* isolates by biotyping tests revealed that 4 strains were identified as *B. melitensis* biovar 3 and 2 strains were identified as *B. melitensis* Rev.1 strain. These results were confirmed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) as a molecular marker technique.

**Key words:** PCR-RFLP, Conventional serological test, *B. melitensis*, Vaccine

**INTRODUCTION**

Brucellosis remain an important disease in both human and domesticated animals in which the main symptom is reproductive failure. A number of characteristics make Brucella species attractive targets for weaponization and the organism remains on the list of CDC category B potential biological warfare agents. In humans, brucellosis caused by *B. melitensis* is by far the most important clinically apparent disease and is usually associated with occupational exposure or the consumption of unpasteurized dairy products. Moreover, *B. melitensis* is one of the major causes of abortions in sheep and goats and the organism is secreted in the milk of infected animals.

*Brucella melitensis* persists in Mediterranean and Middle East countries and sporadically all over the world. It is associated with nomadic animal husbandry which itself is related to developing countries. For this reason a test and slaughter policy is not realistic in the majority of places where *B. melitensis* is endemic due to lack of financial resources needed for compensation. International agencies have, therefore, proposed that whole flock vaccination should precede any test and slaughter programs until disease prevalence is significantly reduced. Only then test and slaughter should be implemented as a part of national eradication scheme.

The Rev.1 vaccine was developed by Elberg and Faunce and has been successfully applied in sheep and goat for the control of ovine and caprine brucellosis. It was recognized that Rev.1 vaccination cause existence of positive reactors in serological tests among vaccinated population which lead to difficulties in distinguishing between infected and vaccinated animals by conventional serological tests. Bacteriological isolation and identification of etiological agent are necessary step in the design of epidemiological and eradication program.

The classical method of identifying the species and biovars of *Brucella* strains requires a minimum of five days. Whereas, the development of genus-specific and species-specific PCR assays for identification has been possible, typing tools of sufficient resolution to permit epidemiological tracing of outbreaks are still lacking. One of the most promising molecular approaches to date utilizes DNA polymorphism is the PCR-RFLP analysis of *Brucella* *omp2* gene.

The aim of this work is the differentiation between Rev.1 vaccine strain and *B. melitensis* biovar 3 field strain using the conventional techniques represented by bacteriological isolation and identification and recent molecular techniques.
represented by PCR-RFLP. We used the omp2 gene as a locus of two nearly homologous repeated copies that differ slightly among Brucella species and biotypes in presence or absence of the Pst I site to differentiate between them[5,11].

MATERIALS AND METHODS

Samples: Serum samples were collected from 32 Egyptian native breed ewes (5-8 months old). Animals were purchased from different areas from Menufia governorate of unknown history of brucellosis or vaccination to be introduced in a private farm in El-Giza governorate for reproduction purpose.

Serological Examination: Standard tube agglutination test (SAT), Rose Bengal plate test (RBPT), Rivanal test (Riv T) and complement fixation test (CFT) were carried out according to Alton et al.[1].

Bacteriological Analysis: Bacteriological culture specimens were evaluated. This included retropharyngeal, supramammary lymph nodes and spleen tissues obtained from animals that were seropositive tests. Specimens were processed using the method described by Alton et al.[5]. Biochemical tests, dye sensitivity, exposure to monospecific antisera, susceptibility to antibiotics and lysis by brucella phages were performed on colonies with characteristic typical of the genus Brucella.

Bacterial DNA Extraction: The bacterial strains used in this study were standard Rev 1 vaccine strain as control, and six brucella isolates cultured from seropositive ewes for brucella infection. The DNA was extracted by Genomic DNA purification kits (Puregene).

Primers Used in Microsatellite Amplification: Brucella-specific primer pairs were designed from brucella omp 2 gene[5]. These primers were synthesized at AGERI on an ABI 392 DNA/RNA synthesizer (Applied Biosystems). The sequence of the forward and reverse primer pairs is given in Table (1).

Polymerase Chain Reaction: PCR amplification was performed according to the method of Mullis and Faloona [15]. 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 albumen (fraction IV; Sigma) 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, 10 pM of each oligonucleotide primer and 0.5 U of Taq polymerase (Promega). Following hot start treatment at 95°C for 3 min., PCR was performed with an Eppendorf thermocycler (Applied Biosystem Gene Amp PCR system 9700) as follows: 35 cycles of PCR with 1 cycle consisting of 20 s at 95°C for DNA denaturation, 1 min at 50°C for primer annealing and 1 min at 72°C for polymerase mediated primer extension. The last cycle included incubation of the sample at 72°C for 7 min. Ten micro liters of the amplified product was analyzed by electrophoresis in ethidium bromide stained 1.5% agarose gels in TEA buffer (20 mM Tris-acetate, 1 mM EDTA [pH 8.0]). The molecular weight estimated by comparison to ÖX174 RF DNA Hae III Digest marker (Biolabs 202-6L). The gels were then photographed using a Polaroid Camera.

Digestion of the Amplified Products: Pst I restriction enzymes was used according to the manufacturer's instruction (Biolabs 1405).

Electrophoresis of PCR-RFLP Products: Two different methods were used to visualize the result of PCR products digestion (a) Agarose gels, where the PCR products were loaded on Ethidium bromide stained 1.5% agarose gel (FMC, Bio products, Rochland, ME). (b) 10% non denaturing polyacrylamide gels in the ProTEAN® IIxi cell system from BioRad were used. Polyacrylamide gels were prepared by adding twenty five ul of TEMED and 350 ul of 10% APS (Ammonium per sulfate) added to 16.6 ml of 30% acrylamide, 5 ml 10XTBE and 28.4 ml H₂O and mixed by swirling. Five ul of each sample with loading dye was loaded into the gel wells and separated in 1X TBE buffer. The gel was gently stained with 0.5 μg/ml ethidium bromide in 1X TBE for 30 min at room temperature. The molecular weight was estimated by ÖX174 RF DNA Hae III digest marker (Biolabs 202-6L), then photographed using a Polaroid Camera.

RESULTS AND DISCUSSIONS

Serum samples were collected from 32 examined ewes and subjected to serological examination for detection of specific Brucella antibodies. From these animals 10 (31.25%), 8 (25.00%), 7 (21.88%) and 7 (21.88%) were positive for SAT, RBPT, Riv T and CFT, respectively (Table 2). Retropharyngeal,

<table>
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<th>Table 1: The sequences of the oligonucleotide primers</th>
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<td>Sequences</td>
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<tr>
<td>Forward P1  5’TGGAGGTCAGAAATGAAC’</td>
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<tr>
<td>Reverse P2  5’ GAGTGGCAACACGAGCGC’</td>
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supramammary lymph nodes and spleen tissue were collected from seropositive animals and subjected to bacteriological examination. *Brucella* organisms were detected in tissue samples collected from six seropositive animals only. All isolates were typical of isolates of *B. melitensis* in morphology, colonial appearance and growth characteristic. None was dependent on CO₂ for growth and none produced more than traces H₂S.

In agglutination test with antisera monospecific for A and M epitopes of *Brucella* smooth lipopolysaccharides antigens, four strains revealed agglutination with A and M monospecific antisera while two strains showed agglutination with M monospecific antisera only.

To distinguish between those isolated *B. melitensis* strains, we evaluated their growth in the presence of penicillin, streptomycin and the dyes basic fuchsin and thionin added at recommended concentration to tryptic (Difco Laboratories) soy agar (Table 3).

The results obtained from these tests showed that four isolates were identified as *Brucella melitensis* biovar 3 and two isolates identified as Rev.1 melitensis strain.

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**Table 2:** Serological and bacteriological Results of samples collected from examined ewes:

<table>
<thead>
<tr>
<th>Samples No.</th>
<th>SAT(+)</th>
<th>RBAT(+)</th>
<th>RivT(+)</th>
<th>CET(+)</th>
<th>Culture</th>
<th>Samples No.</th>
<th>SAT(+)</th>
<th>RBAT(+)</th>
<th>RivT(+)</th>
<th>CET(+)</th>
<th>Culture</th>
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</tbody>
</table>

**Table 3:** Results of identification at the biovar level of *Brucella melitensis* isolates recovered from serologically positive ewes

<table>
<thead>
<tr>
<th>Recovered strain</th>
<th>No of isolates</th>
<th>CO₂ requirement</th>
<th>H₂S production</th>
<th>Urease test</th>
<th>Growth on dye</th>
<th>Monospecific- sera</th>
<th>Growth on media with antibiotics</th>
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<tbody>
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<td>--</td>
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<td>+</td>
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<td>Rev .1</td>
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* a= antibody titer  b= degree of agglutination  ND= Not Done

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Fig. 1: Agarose gel electrophoresis of PCR-amplified omp 2 gene fragments from Brucella strains. The figure shows a single band 282-bp DNA fragment. M: ØX 174 RF DNA HaeIII digest marker (Biolabs). Lane 1, standard B. melitensis Rev. 1 vaccine strain. Lanes: 2, 4, 6 and 7, B. melitensis biovar 3 field strain. Lanes: 3 and 5 B. melitensis Rev.1 strain isolated from ewes.

Fig. 2: Agarose gel electrophoresis of Pst I digest of amplified omp 2 gene fragments from Brucella strains. The figure shows the 282 pb DNA lacking the pstI site, and the large, Pst I digested DNA fragments. The smaller, 44-bp DNA fragment is not shown. M: ØX 174 RF DNA HaeIII digest marker (Biolabs). Lane: 1 standard B. melitensis Rev. 1 vaccine. Lanes: 2, 4, 6 and 7, B. melitensis biovar 3 field strain. Lanes: 3 and 5, B. melitensis Rev. 1 strain isolated from ewes.

The PCR test was performed with B. melitensis standard strain Rev. 1 and B. melitensis biovar 3, field strain and B. melitensis Rev 1 strain isolated from ewes. A single band with the expected size of 282 bp was obtained with all isolates as shown in Fig. (1).

Digestion of the amplified fragments from the obtained strains with Pst I restriction endonuclease gave different bands on agarose gels (Fig. 2). B. melitensis standard Rev. 1 vaccine strain digests

Fig. 3: Polyacrylamide gel electrophoresis of Pst I digests of amplified omp 2 gene fragments, the 282bp fragment DNA and the two Pst I digested DNA fragments with sizes 238 and 44 bp, respectively. Lanes: M, ØX 174 RF DNA HaeIII digest marker (Biolabs). Lane: 1, standard B. melitensis Rev. 1 vaccine. Lanes: 2, 4, 6 and 7 B. melitensis biovar 3 field strain. Lane: 3 and 5, B. melitensis Rev.1 strain isolated from ewes.

(lane 1) revealed two visible bands: a large band, which was uncut DNA, 282 bp. Due to the lack of Pst I site and another band 238-bp size. In comparison, the Pst I digestion of 4 B. melitensis isolated strain digests (lanes 2, 4, 6 and 7) revealed a single band, 238-bp in size, while, the two other strains digest gave two visible bands similar to that of the vaccine strain (lanes 3 and 5).

The profile of the digested PCR products DNA were analyzed by polyacrylamide gel electrophoresis, as shown in Fig (3). The purpose of this analysis was to visualize the smaller fragment that was not shown by agarose gel electrophoresis. In Fig (3), besides the 282- and 238-bp DNA bands, all samples produced an additional identical smaller fragment which was calculated to be 44-bp. It was calculated that the two small bands (44 and 238 bp) together were the same size as the uncut DNA.

Discussion: Control of brucellosis depends mainly upon elimination of infected animals. The most effective plan for elimination of the disease is the detection of infected animals by periodic testing of milk or blood for specific antibody and elimination of positive reactors[16].

Diagnosis of brucellosis in animals is based mainly on clinical signs, serological and bacteriological investigations. In the present study different serological
tests were used including SAT, RBPT, Riv T and CFT.

The obtained results revealed that in SAT, RBPT, Riv T and CFT, 10 (31.25%), 8 (25.00%), 7 (21.88%) and 7 (21.88%) were positive respectively (Table 2). The higher number of SAT reactors compared to RBPT may be attributed to the higher sensitivity of this test to the IgM than to IgG or may be due to that RBPT is not sensitive enough to detect low antibody titer of some chronically infected animals[14].

Riv T and CFT detected only 7 positive cases each. This might be explained as that Riv T is useful in detection of chronic cases that mainly contain IgG[2].

As well as Salem et al.[20] stated that CFT is the superior one among the employed tests, so for accurate diagnosis of brucellosis it is important to use more than one test including the CFT. Bacteriological examination of serologically positive animals revealed the recovery of Brucella isolates from only 6 seropositive cases from retropharyngeal, supramammary lymph nodes and spleen tissue. This indicated that the sensitivity of serological test was higher than that of the culture method. The same conclusion was reached by Hamdy and Amin[13] who suggested that the most specific diagnostic test involves isolation of the causative organism, but this suffers from the drawback of requiring a long incubation period and low sensitivity especially in the chronic stage of the disease. Moreover, the culture material must be handled carefully as the Brucella organism is a class III pathogen.

Only six Brucella melitensis isolates were identified from these examined animals, 4 isolates identified as B. melitensis biovar 3 and 2 isolates as B. melitensis Rev 1 strain (Table 3). The isolation of B. melitensis Rev 1 strain from these animals obtained from different localities of unknown history of infection or vaccination may be due to previous vaccination with Rev. 1 vaccine or due to Rev. 1 horizontal infection[3]. These results confirmed the findings of Pefanis et al.[17] who reported that subcutaneous vaccination with Rev. 1 vaccine in sheep produces an intense serological response as measured by standard serological tests which doesn't permit distinction between vaccinated and infected animals.

Moreover, Pieterson et al.[18] suggested that the use of live vaccine, albeit attenuated, raised specific concern about the capacity of the strain to infect individual human subjects and the potential risk of being horizontally transferred in the field. To circumvent these problems the national veterinary services need to maintain a certain level of competence. In this context, they need to design a method to identify and register vaccinated animals and distinguish them from infected ones. In the laboratory the serological tests had to be performed according to standard criteria and infection status should have pre-determined. Finally bacteriological samples must be sent to the laboratory for strain isolation and biotyping.

The PCR technique has increasingly been used as a supplementary method in Brucella diagnosis[12]. Recently, a molecular biotyping approach has been proposed on the basis of restriction endonuclease polymorphism in the genes encoding the major 25- and 36-KDa outer membrane proteins of Brucella[7]. The omp 2 gene exists as a locus of two nearly homologous repeated copies(omp2a and omp2b) that differ slightly among Brucella spp.[11]. We used this information to design specific primers that amplify a 282-bp fragment (Fig.1), flanking upstream sequences of the 5 terminus of the two genes (omp2a and omp2b) and expanding downstream of the Pst I sites[10]. The sensitivity of the test would be doubled by selecting duplicated DNA sequences of the two genes. Moreover, because of the existing Pst I site polymorphism between brucella strains, the test distinguish between the vaccine strain and field strain of Brucella melitensis.

Our results revealed that DNA fragments obtained from B. melitensis standard Rev 1 vaccine strain and two isolates from seropositive ewes identified as B. melitensis Rev 1 strain produce three bands, an intact 282-bp fragment from the amplified omp2a gene that lacks the Pst I site and two smaller fragments of 238 and 44 bp, the product obtained from digestion of the omp2b amplified fragment. In contrast B. melitensis field isolate (B. melitensis biovar 3) produced only two smaller fragments from both genes(omp2a and omp2b), a 238-bp fragment and a 44-bp fragment. This results came in close agreement to that described by Ficht et al.[10] and Bardenstein et al.[9].

Also, our results are in good agreement with those of Cloechaert et al.[7] who mentioned that B. melitensis isolates were split between those with a single Pst I site located in the omp2b gene (B. melitensis Rev 1 strain) and those with two Pst I sites, one in omp2a and one in omp2b (B. melitensis biovar 3).

B. melitensis strain isolated from two ewes (as shown in lanes 3 and 5 Fig. 2 and 3) gave similar pattern of Pst I digestion profile of standard vaccine
strain. This indicates that these animals were previously vaccinated with Rev 1 vaccine or horizontally infected with vaccine strain. This has been previously cited by Bardenstein et al.[5] and Banai et al.[4] who demonstrated that the Rev 1 vaccine led to the adverse effects of strain persistence in the vaccinated animals and was occasionally spread horizontally. In two cases it was shown that the vaccine strain caused human infection, demonstrating the zoonotic hazards of its virulence.

In this study, PCR-RFLP was shown to be a valuable tool for differentiating *B. melitensis* Rev. 1 vaccine strain from *B. melitensis* biovar 3 field strain in less than 24 hours. Moreover, it is more sensitive and more specific in comparison to the conventional techniques and it can be used in clinical samples directly. The conventional methods of identification require a minimum of 5 days to identify an isolate to *Brucella* species and biovar level. This can delay the movement of animals between different owners and have a negative impact on the owners' financial planning. This study indicates that Brucellosis eradication program personnel could reliably use the abbreviated brucella PCR-RFLP to supplement other diagnostic and epidemiological data (such as herd history and serological test results) to release sale animals from quarantine before the conventional identification methods are completed.

**REFERENCES**