Isolation and Biotyping of *Brucella melitensis* from Upper Egypt

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**Abstract:** A total 106 seropositive samples from sheep, cattle and goats were collected from May 2009 to May 2010. Species of *Brucella* were isolated from, 9 (28.13%) of 32 in cattle, 30 (40.5%) of 74 in sheep and goats, from lymph nodes and spleen tissues. The south province of Egypt. The species examined by biochemical characteristics and had identical reactions with the standard strain. Oxidative metabolic tests performed, by substrate specific tetrazolium reduction (SSTR) test on the species, confirmed them as *B. melitensis*. Based on the biochemical, oxidative metabolic, and biotyping tests (CO₂ requirement, H₂S production, growth in the presence of thionin and basic fuchsin dyes, and agglutination test with monospecific A and M anti-sera) the strains were determined as *B. melitensis* biotype 3.


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**Keywords:** *Brucella melitensis*; Isolation; Biotyping; Upper Egypt

1. Introduction

Brucellosis is a zoonotic disease which affects several species of domestic animals commonly reared by humans for the production of milk, meat, and wool. Because of the complications involved in disease diagnosis, including the difficulties in distinguishing between infected and vaccinated animals by conventional serological tests (Alton, 1980, Diaz et al., 1979), bacteriological isolation and identification of the etiological agent are necessary steps in the design of epidemiological and eradication programs (Plommet, 1986, Refai, 2002, Zinstag et al., 2005).

Molecular diagnostic methods are also currently being used for the detection of *Brucella* sp. in various materials. The diagnosis of brucellosis in sheep and goats is based on serological, bacteriological, allergic, and molecular methods (Simsek et al., 2004).

*B. melitensis* is one of the major causes of abortion in sheep and goats and is secreted in the milk of infected animals. The transmission of *B. melitensis* to cattle, buffaloes, and camels is now the predominant cause of brucellosis in animals and humans in most Middle Eastern countries (Al-Majali, 2005, Al-Talafih et al., 2003, Ocholi et al., 2005, Refai, 2002). *Brucella melitensis* is the main etiological agent of brucellosis in sheep and goats, and is also the main agent responsible for human brucellosis, a predominantly occupational disease related to professions in direct contact with livestock (Blasco and Molina-Flores, 2011).

*Brucella* species are highly monomorphic, with minimal genetic variation among species (Tiller et al., 2009) and maintain a close taxonomic relationship and can only be distinguished by rigorous metabolic, immunologic, and biochemical analyses. The similarities among the *Brucella* species extend to the genetic level at which all species share greater than 90% DNA homology (Hoyer and McCullough, 1968 (a), (b)). Species of *Brucella* were differentiated in the laboratory by colonial morphology, growth requirement, various biochemical tests and lysis by bacteriophage (Christina, 1998).

Oxidative metabolism tests were done on selected strains to confirm the species identification by phage typing (Corbel and Brinley-Morgan, 1984). In addition, oxidative metabolic patterns accurately identify the species in this genus, and that by the conventional methods of differentiation, many strains of *B. abortus* are misidentified as *B. melitensis* (Meyer, 1961). The accurate distinction between *Brucella* species and their biovars is performed by differential tests based on phenotypic characterization of lipopolysaccharide antigen, phage typing, dye-sensitivity, CO₂ requirement, H₂S production and metabolic properties (Alton et al., 1988). Available information indicates that *B. melitensis* infection is mostly widespread in Egypt, Sudan, Syria, Morocco, Turkey, Greece, Spain, and Italy, and in some Latin American countries (Benkirane, 2006, Minas, 2006, Refai, 2002).

The present study aimed to isolate *Brucella* sp. from sheep, cattle and goats by using standard cultural methods, and to biotype these isolates in order to establish a epidemiological base for studies on the
control and prevention of brucellosis in Assuit governorate.

2. Material and methods
This study was conducted during the years 2009 and 2010 in the south province of Egypt (Assuit governorate) and the tests were performed on all field and standard strains (B. abortus 544, B. melitensis 16M and B. suis 1330 originally provided by AHRI).

2.1. Brucella isolation
The isolates discussed in this study are described in Table 1. Brucella from seropositive animal cultures were isolated in Animal Health Research Institute (AHRI) laboratory by the methods of Alton et al. (1988).

2.2. Bacteriological examinations.
All obtained tissues cultured on Brucella agar selective media (Oxoid) at 37°C in presence of 10% CO₂ for up to 2 weeks. The suspected colonies were examined for Brucella sp. Brucella-suspected colonies were characterised by the morphology, Gram stain, oxidase, catalase, urease production, and nitrate reduction tests (Sahin, et al., 2008). Colonial phase and staining were studied by, agglutination in acriflavine, crystal violet, and Zehil-Neelson staining. In addition, motility and serum requirements.

2.3. Metabolic characteristics.
Oxidative metabolic studies were conducted by using substrate specific tetrazolum reduction (SSTR) test (Broughton and Jahans, 1997, Ewalt et al. 2001), and the substrates used were previously reported in Ewalt and Forbes (1987) in addition to uroconic acid.

2.4. Biotyping tests.
The CO₂ requirement, H₂S production, growth in the presence of thionin (1:25,000, 1:50,000, and 1:100,000 dilutions) and basic fuchsin (1:50,000, and 1:100,000 dilutions) dyes, and agglutination with monospecific A, M and R anti-sera, were performed as the methods of Alton et al. (1988).

3. Results

Brucella isolation.

Brucella sp. was isolated from different lymph nodes and spleen tissues was of 9 (28.13%) out of 32 in cattle, and 30 (40.5%) out of 74 in sheep and goats, while the overall rate of isolation was 36.8% of the total number of examined animals.

Species identification and biotyping
The results obtained in Table 2 revealed identification at the Brucella genus of 39 field isolates compared to reference strains by their colonial morphology, staining, serum requirement, motility and biochemical reactions. Suspected resultant colonies were further identified as Brucella sp. by the morphological appearance of each colony and microscopic appearance according to Alton et al. (1988) where, all cultures isolated from different animal species were characterized. The culture smears showed Gram-negative coccobacilli in Gram's staining. The colonies were round, convex, smooth margin, translucent, hony-colored, glistenining, and bulish on Brucella selective media. There was no agglutination with acriflavine, and not stain with crystal violet staining.

The cultures were positive for biochemical reactions (catalase, oxidase, nitrate reduction, and urease tests). There are some variation in urease activities shown between reference strains, rabid, slow, and moderate in Br. suis, Br. abortus, and Br. melitensis, respectively. Moreover, positive urease activity was observed on Christensen's medium.

In oxidative metabolic studies (Table 3), both field and standard Brucella strains utilized the substrates, amino acids (D-alanine, L-alanine, L-asparagine, and L-glutamic acid), carbohydrates (L-arabinose, D-galactose, D-ribose, D-glucose, and Meso-erythritol), and didn't utilize, urea cycle amino acids, uroconic acid and L-arabinose.

From the growth pattern on basic fuchsin, thionin, the dominant M and A antigen, non requirement of carbon dioxide and non production of H₂S in Table (4), the Brucella strains identified as B. melitensis. Based on the results in Table 2, 3 and 4, biochemical tests, morphology and agglutination test with monospecific A and M antisera, all the Brucella field isolates were determined as B. melitensis biovar 3. This finding is consistent with reports of B. melitensis, particularly biovar 3, being the main cause of brucellosis in animals among Assiut governorate.

Table 1: Brucella sources and isolation percentages

<table>
<thead>
<tr>
<th>Brucella source</th>
<th>Sample number</th>
<th>Isolate number</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal species</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cattle</td>
<td>32</td>
<td>506</td>
<td>39</td>
</tr>
<tr>
<td>Sheep and goats</td>
<td>74</td>
<td>356</td>
<td>74</td>
</tr>
<tr>
<td>Total</td>
<td>106</td>
<td>506</td>
<td>106</td>
</tr>
</tbody>
</table>

* Five lymph nodes for each carcass including tetropharyngial, prescapular, pteremural, internal iliac, and supramammary.
Table 2. Morphological and Bacteriological examinations of *Brucella* isolates.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Colonial morphology</th>
<th>Colonial phase &amp; staining</th>
<th>Biochemical reactions **</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Indirect inspection</td>
<td>Direct inspection</td>
<td>Serum requirements</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Catalase</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Oxidase</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Urease</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nitrate reduction</td>
</tr>
<tr>
<td><strong>Total number of isolates/host</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9/cattles</td>
<td>Round, smooth, translucent and honey-coloured</td>
<td>No agglutination</td>
<td>-</td>
</tr>
<tr>
<td>25/sheep</td>
<td>Round, glistening, and bulish</td>
<td>No staining</td>
<td>+</td>
</tr>
<tr>
<td>5/goats</td>
<td>Modified ZN*</td>
<td>Gram negative coccobacilli</td>
<td>Weak acid fast</td>
</tr>
<tr>
<td><em>Reference strains</em></td>
<td></td>
<td></td>
<td>Non motile</td>
</tr>
<tr>
<td><em>Br. melitensis</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Br. abortus 544</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Br. suis 1330</em></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ziehl-Neelsen stain. **Results:** - negative, + positive, ++ strong positive.

Table 3. Oxidative metabolic profiles* of *Brucella* spp.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Substrate** groups</th>
<th>Amino acid</th>
<th>Carbohydrate</th>
<th>Urea cycle amino acid</th>
<th>Uricolic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td><strong>Number of <em>Brucella</em> spp./host</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Reference strains</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Optical density with substrate/Optical density with no substrate = 1-3; 1 = 3-5; 2 = 6-8; 3 = 9-12. **Substrates:** A-L-alanine; B-L-asparagine; C-L-glutamic acid; D-L-arabinose; E-D-galactose; F-D-ribose; G-D-glucose; H-D-xylose; I-Mesoerythritol; J-L-arginine; K-DL-ornithine; and L-L-lysine.
Table 4. Biotyping tests of *Brucella melitensis* strains.

<table>
<thead>
<tr>
<th>Culture</th>
<th>CO₂ requirements</th>
<th>H₂S production</th>
<th>Growth on dye</th>
<th>Nonspecific antisera</th>
<th>Biotype metabolic pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Thionin A</td>
<td>Thionin b</td>
<td>Fuchsine C</td>
</tr>
<tr>
<td>9/Cattle</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>25/Sheep</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5/Goats</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Number of <em>B. melitensis</em> field strains/host</td>
<td></td>
<td></td>
<td>Br. melitensis bv 3</td>
<td></td>
<td>Br. melitensis 16M</td>
</tr>
<tr>
<td>Br. melitensis 16M</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Br. abortus 544</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Br. suis 1330</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

**Abbreviations:** a-Dye concentration 1:25,000(40ug/ml); b-Dye concentration 1:50,000(20ug/ml); c-Dye concentration 1:100,000(10ug/ml); A-Monospecific antisera; M-Monospecific antisera; R-Rough Brucella antisera.

**Discussion**

Brucellosis is a worldwide zoonotic disease that is recognised as a major cause of heavy economic losses to the livestock industry and poses serious human health hazard (Ocholi *et al.*, 2005). *B. melitensis* is the main aetiological agent of brucellosis in small ruminants. Ewes' and nanny-goats' aborted foetuses and products derived from sheep and goats remain the main source of infections. The results of *Brucella* isolation from different lymph nodes and spleen tissues were agree with (Esmaeil *et al.*, 2008, Sahin *et al.*, 2008, Aras and Ateş, 2011), while the overall rate of isolation was also agree with, Cvjetnić *et al.* (2009) isolated *Brucella* from 88 out of 151 serologically positive pigs (58.3%) and 7 of 93 (7.5%) wild boar, Al-Farwachi *et al.*, 2010 isolated from 4 (33.3%) of 12 samples, and Muñoz *et al.*, 2010 recovered 104 isolates (19.3%) were obtained from seropositive animal cultures. In contrast, one (12.5 %) of 41 bovine abortion cases was *B. melitensis* biotype (Table 1).

The results obtained in Table 2 revealed identification at the *Brucella* genus of the field isolates compared to reference strains. There was no agglutination with acriflavine, and not stain with crystal violet staining as reported recorded in Songer and Post (2005). The cultures were positive for biochemical reactions (catalase, oxidase, nitrate reduction, and urease tests). Similarly, Corbel and Brinley-Morgan (1984), Carter and Cole (1990) reported all *Brucella* strain were oxidase, catalase positive and can reduces nitrates to nitrite, serum (not required), and non-motile. They are not truly acid-fast but resist discoloration by weak acids, and stain red by the Stamp's modification of Ziehl-Neelsen method (Lennette *et al.*, 1985) as well as reference strain. Moreover, positive urease activity was observed on Christensen's medium. From the details in Table (2) all isolates are compatible with, those described for the genus *Brucella* (Alton *et al.*, 1988) and belonging to the *Brucella* organisms, and with that obtained (Leyla *et al.*, 2003, Mantur *et al.*, 2004, Unver *et al.*, 2006 and Helmy *et al.*, 2007).

The differentiation of *Brucella* species by substrate specific tetrazolium reduction (SSTR) test has been carried out by Broughton and Jahans (1997), and Ewalt *et al.* (2001). Based on oxidative metabolic studies (Table 3), all field *Brucella* species identified as *Br. melitensis*, their behavior on the substrates are agree with those reported by Broughton and Jahans (1997) and Ewalt *et al.* (2001). *Brucella melitensis* is a major human and animal pathogen, with a wide host range that includes all domestic ruminant species, although small ruminants are its preferred hosts (Álvarez *et al.*, 2011). From the growth pattern in Table (4), the *Brucella* strains identified as *B.*
melitensis. These results are combatable with the identified B. melitensis reference strain and agree with the reported (Meyer and Shaw, 1984, Ewalt et al. 1987, and Songer and Post, 2005).

The existence of different Brucella biotypes among the Brucella species facilitated the identification of the source of the infection (Guler et al., 2003). Based on the results in Table 2, 3 and 4, biochemical tests, morphology and agglutination test with monospecific A and M antisera, all the Brucella field isolates were determined as B. melitensis biovar 3. These results coincide with those reported by (Buyukcangaz and Sen, 2007, and Sahin et al., 2008, Aras and Ateş, 2011). This finding is consistent with reports of B. melitensis, particularly biovar 3, being the main cause of brucellosis in animals among Assiut governorate. B. melitensis biovar 3 from cows, ewes and goats also isolated in Assiut (Salem, et al., 1987). Moreover, Br. melitensis has epidemiological and zoonotic important as this strain of the most pathogenic strain to animals in Assuit (Ali et al., 1993). Isolation of Br. melitensis biovar 3 from, sheep and goats (Sayour et al., 1970 and El-Bayoumy, 1989), and cattle (El-Gibaly, 1969, Sayour et al., 1970, Montasser, 1991, and Helmy et al., 2007) was also recorded in Egypt.

In conclusion, the isolation and biotyping of Br. melitensis particularly biovar 3, the most pathogenic strain and the main cause of brucellosis in different animals species among Assiut governorate, is a very dangerous alarm and gives spot light for application of preventive hygienic measures and control program of Brucella not only in upper but in all Egypt.

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References


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