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 (CO_2 requirement, H_2S 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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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-Talafhah 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, CO_2 requirement, H_2S 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% CO_2 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 CO_2 requirement, H_2S 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 coccobacili in Gram's staining. The colonies were round, convex, smooth margin, translucent, hony-coloured, 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 H_2S 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* biovare 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

Brucella source		Sample number		Isolate number	Percentage (%)
Animal species	Number	Lymph nodes*	Spleen		
Cattle	32	150	32	9	28.13
Sheep and goats	74	356	74	30	40.5
Total	106	506	106	39	36.8

** Five lymph nodes for each carcass including tetropharyngial, prescapular, ptefemural, internal iliac, and supramammary.*

Table 2. Morphological and Bacteriological examinations of *Brucella* isolates.

Culture		Colnial morphology		Colonial phase & staining				Motility	Serum requierments	Biochemical reactions **			
		Indirect inspection	Direct inspection	Acriflavin test	Crystal violet	Gram's staining	Modified ZN*			Catalase	Oxidase	Urease	Nitrate reduction
Total number of isolates /host	9/cattles	Round, convex, 1-2mm. in diameter, smooth margin, translucent and honey-coloured	Round, glistenining, and bulish	No agglutination	No staining	Gram negative coccobacilli	Weak acid fast	Non motile	-	+	+	+	+
	25/sheep								-	+	+	+	+
	5/goats								-	+	+	+	+
Reference strains	*Br. melitensis* 16M								-	+	+	+	+
	Br. abortus 544								-	+	+	-	+
	Br. suis 1330								-	+	+	++	+

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

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

Culture		Substrate** groups												Urocanic acid
		Amino acid			Carbohydrate						Urea cycle amino acid			
		A	B	C	D	E	F	G	H	I	J	K	L	
Number of *Brucella* spp. /host	9/Cattle	3	1	2	-	1	2	3	2	3	-	-	-	-
	25/Sheep	3	2	2	-	2	1	2	2	2	-	-	-	-
	5/Goats	1	2	1	-	3	2	3	2	3	-	-	-	-
Reference strains	*Br. abortus* 544	1	1	2	1	2	3	2	1	3	-	-	-	1
	Br. melitensis 16M	2	2	2	-	2	1	2	1	2	-	-	-	-
	Br. suis 1330	1	1	2	3	2	3	2	3	1	2	2	2	2

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.

Culture		CO_2 requirements	H_2S production	Growth on dye					Nonspecific antisera			Biovare metabolic pattern
				Thionin			Fuchsin		A	M	R	
				A	b	C	a	b				
Number of *B. melitensis* field strains/ host	9/Cattle	-	-	-	+	+	+	+	+	+	-	*Br. melitensis bv3*
	25/Sheep	-	-	-	+	+	+	+	+	+	-	
	5/Goats	-	-	-	+	+	+	+	+	+	-	
Reference strains	*Br. melitensis* 16M	-	-	+	+	+	+	+	-	+	-	1
	Br. abortus 544	+	+	-	-	+	+	+	+	-	-	1
	Br. suis 1330	-	-	-	+	+	-	-	+	-	-	1

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 aetiologic 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, Cvetnić 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* biovare 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 zoontic 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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