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, 25 (36.23%) out of 69 in sheep and of 5(100%) out of 5 in 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.

[Affi, M. M., Abdul-Raouf, U. M., El-Bayoumy, E. M., Montasser, A. M. and Mohamad, H. A. **Isolation and Biotyping of *Brucella melitensis* from Upper Egypt.** Journal of American Science 2011;7(3):653-659]. (ISSN: 1545-1003). http://www.americanscience.org.

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

1. Introduction

The most reliable and the only unique method for diagnosing animal brucellosis is isolation of *Brucella* species (Alton *et al*., 1988). A definite diagnosis requires the isolation of *Brucella* sp. from blood, bone marrow or other tissues. However, cultural examinations are time-consuming, hazardous and not sensitive. Thus, clinicians often rely on the indirect proof of infection (Al-Dahouk *et al*., 2003; Bounaadja *et al*., 2009), bacteriological isolation and identification of the etiological agent are necessary steps in the design of epidemiological and eradication programs (Refai, 2002, Zinstag et al., 2005).

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). 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).

The comprehensive testing of metabolic activity allows cluster analysis within the genus *Brucella*. The biotyping system developed for the identification of *Brucella* and differentiation of its species and biovars may replace or at least complement time-consuming tube testing especially in case of atypical strains (Al-Dahouk et al., 2010).

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. Materials 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*s 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, 25 (36.23%) out of 69 in sheep and of 5(100%) out of 5 in 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.

4. Discussions

Brucellosis is a worldwide zoonotic disease that is recognized 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. Ovine and caprine brucellosis were reported as a most common epidemic infection in Mediterranean and Middle Eastern countries, Asia, Latin America, and South Europe (Minas, 2006, Refai, 2002).

The studies in various parts of Egypt indicate that the *Br. melitensis* biovar 3 isolated from sheep, 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). Confirmatory diagnosis must be provided by the isolation of aetiological agents. Therefore, the isolation of *B. melitensis* is important to study the epidemiology of brucellosis.

The isolation of 39 *B. melitensis* strains from 106 (32 in cattle, 25 in sheep and 5 in goats) may indicate very high prevalence of *B. melitensis* infection among these animals in this region and due to that, the disease may threat human and animal health which was coincide (Esmaeil et al., 2008, Sahin et al., 2008, Aras and Ate , 2011).

Limited reports are available on the identification and biotyping of *B. melitensis* in Assuit. Salem, et al. (1987) and Ali et al. (1993) indicate that the disease is widespread among cows,

ewes and goats and isolated *B. melitensis* biovar 3. The overall rate of isolation was 36.8%. Cvetni 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.

The application of biochemical tests are used for the identification of *Brucella* sp. The isolates in the present study 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) which was similar to the biochemical test reported (Leyla et al., 2003, Mantur et al., 2004, Songer and Post, 2005, Unver et al., 2006, Helmy et al., 2007).

Although *Brucella* is a monophyletic genus, apparent differences between its species do exist e.g. host specificity and pathogenicity. Nowadays, *Brucella* species and biovars are distinguished by a limited number of microbiological tests measuring quantitative or qualitative differences of dye bacteriostasis, hydrogen sulfide production, urea hydrolysis, carbon dioxide requirement, bacteriophage sensitivity and agglutinin absorption as carried reported (Broughton and Jahans, 1997, and Ewalt et al., 2001) by substrate specific tetrazolium reduction (SSTR).

For at least half a century these microbiological procedures have not changed, although various new *Brucella* species showing variable phenotypic traits have been detected and new diagnostic methods have been developed. Neither the classical biochemical tests nor antigenic properties and phage-sensitivity can be considered a reliable guide to the identification of *Brucella* species. Contradictory results were often reported (Meyer and Morgan, 1962). However, variations in H_2S production, CO_2 requirement, a change in dye tolerance or atypical surface antigens i.e. inconsistent A and M antigens usually do not affect the oxidative metabolic pattern of a strain (Cameron and Meyer, 1958, Wundt, 1963).

Metabolic activities have proven to be stable parameters allowing unambiguous species identification, particularly in strains which show conflicting identities by conventional determinative methods (Meyer, 1961a, Meyer 1961b, Meyer 1962). Using the most discriminating carbon substrates i.e. D-glucose, D-trehalose, D-ribose, palatinose, L-fucose, L-malate, and DL-lactate more than 80% of the *B. melitensis* and *B. abortus* strains could be correctly identified (Al-Dahouk et al., 2010).

In our series, all field *Brucella* species identified as *Br. melitensis,* displayed an atypical metabolic pattern could be identified. Oxidative metabolic profiles remain qualitatively stable for long periods of time and usually show no change in characteristic patterns after in vivo and in vitro passages (Jensen et al., 1996).

Comprehensive metabolic studies including all currently known species and biovars are rare. Using 13 differentially oxidized substrates, *Brucella* spp., could be grouped into *Br. melitensis*, their behavior on the substrates are identical with the presently recognized species (Broughton and Jahans (1997), Ewalt et al. (2001), Álvarez et al., 2011). However, *Br. melitensis* strains were tested and biovars were differentiated as biovar 3, which included all of the thirty nine *Brucella.* which was coincide with those reported by (Buyukcangaz and Sen, 2007, and Sahin et al., 2008, Aras and Ate , 2011).

The limited number of field isolates tested per species may have produced inconclusive results, particularly when only reference strains were available which are well known for atypical phenotypic traits. Future studies on larger strain collections may reveal more unique metabolic profiles suitable for species and biovar differentiation.

5. Conclusion:

In conclusion, the comprehensive testing of biochemical, metabolic activity allows cluster analysis within the genus *Brucella*. The biotyping system developed for the identification of *Brucella* and differentiation of its species and biovars may replace or at least complement time-consuming tube testing especially in case of atypical strains.

The isolation and biotyping of *Br. melitensis* particularly biovar 3, the most pathogenic strain and the main cause of brucellosis in some animal 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.

Acknowledgements:

The authors thankful to Professor Ashraf Sayour, Department of Brucellosis, RAHI, Cairo, for the facilities provided in SSTR assay.

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/ 69	336	69	25	36.23
Goats/5	20	5	5	100
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
				Thionin			Fuchsin		A	M	R	
				A	b	C	a	b				
Number of *B. melitensis* field strains/	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.

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3/1/2011