Prevalence of Brucellosis in cattle in Sharkia Governorate

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Abstract
This study was carried out at Sharkia Governorate on 4772 cows (heifers, pregnant and delivered) (2802 located at private farms and1970 individual cases) to estimate the annually prevalence of brucellosis from 2008 to 2011 by using the following tests Buffered Acidified Plate Antigen (BAPA), Rose Bengal Plate (RBP), Serum Tube Agglutination test (STAT) and Rivanol test (Riv.T), Complement fixation test (CFT) and enzyme linked immunosorbent assay (ELISA). The prevalence brucellosis among cattle in various localities was studied and discussed. The total seroprevalence was 4.42 % and 8.9 % for private farms and individual animals respectively serologically during the period from 2008 - 2011. Bacteriological examination of milk samples and lymph nodes tissues collected from reactor animals revealed that *Br. melitensis biovar- 3* was detected by using polymerase chain reaction (PCR). It could be concluded that efforts should be focused on the problem of the disease in cattle who play a role in disease transmission to eliminate it and reduce the prevalence of the disease among cattle.

Key words: *Brucellosis, serodiagnosis , isolation, PCR .*

Introduction

Brucellosis is a wide spread disease among animals and human and of a major economic importance due to abortions, decrease milk yield and temporarily or occasionally permanent sterility **(Nicoletti, 1980)**.

In Egypt, *Br. abortus* was the commonly isolated species since 1970 **(Sayour *et al.*, 1970)**. In the last years *Br. melitensis* became the most common strain prevalent in animals in Egypt **(El Gibaly, 1993, Sayour, 1995, Kadry, 1996** and **Montasser *et al.,* 2002)**.

Brucella microorganism is a facultative, intracellular, gram-negative, bacterial pathogen and the etiologic agent of brucellosis, important zoonoses with a nearly worldwide distribution **(Boschiroli *et al.*, 2001)**.

The distribution of the disease appears to be correlated with high animal densities associated with winter feeding **(Etter and Drew, 2006)**. Infected and susceptible animals commingling on feed grounds ensure exposure of animals to *Br. abortus*, enhancing the probability of transmission. Control of brucellosis should be focused on these sites to prevent or reduce exposure of the pathogen to native animals, thus breaking the chain of transmission.

Clinical symptoms of brucellosis are non-specific and its diagnosis is currently based on serological and microbiological tests **(Alton 1990** and **Díaz-Aparicio *et al.,* 1994)**. While there is no treatment of choice for animal brucellosis,

Bacteriological isolation of *Br. melitensis* and/ or positive blood culture soon after the infection are common laboratory procedures that are used for diagnosis. However, these procedures are not always successful as they are complicated and represent a great risk of infection for laboratory technicians **(Lòpez- Merino, 1991** and **Radostits *et al.,* 2007)**. Serological tests can also be used for diagnosis of *Brucella spp*. infection via detection of antibodies in serum **(Taylor and Francis, 2004** and **Nashowa *et al.,* 2007)**.

In addition, the organism can be detected by polymerase chain reaction (PCR) in blood, semen and abomasal fluid of aborted fetuses and

in comparison to culture method, PCR has more sensitivity and specificity **(Leal-Klevezas and Lòpez-Merino 1995** and **Soliman 2006)**.

Recently, the PCR assay has been used for detection of *Brucella Spp*. It is a promising alternative for conventional bacteriological techniques due to its speedy, safety, high sensitivity and specificity.

The milk ELISA test applied for detecting *Brucella* antibodies in lactating cows was comparatively more sensitive and superior than milk ring test (MRT) **(Biancifiori *et al*., 1996** and **Chand *et al.,* 2002)**, therefore it would be important to examine milk of cows by milk ELISA test.

In Egypt, control of brucellosis is yet a difficult task since it had been diagnosed by **Ahmed (1939)**, despite the exhaustive efforts and difficult concepts of approach, this difficulty is mainly due to the very high coast and the wide range of maintenance factors of *Brucella* organisms, so the current study was carried out to determine the prevalence of brucellosis in Sharkia governorate among cattle by isolation and serotyping of isolates by using PCR.

Material and Methods

Material

Animals:

A total number of 4772 cows (heifers, pregnant and delivered) from year 2008 to 2011, number of animals were 2802 in private farms and 1970 individual randomly spread in the villages. The animals in private farms were kept under restricted program for controlling the internal and external parasites, vaccination programs and standard level of nutrition, whereas mineral mixture and water were available with add libitum. Individual animals were randomly distributed and programs for controlling internal and external parasites and vaccination programs randomly applied.

Samples:

Blood samples:

Blood samples were collected from some private farms and individual distributed animals on different districts in Sharkia province to survey the prevalence of brucellosis and serum were kept in deep freezer at -20°C till used for serological identification.

Milk:

Milk samples were collected from dairy cows serological reactors cows.

Lymph nodes:

Supra mammary internal iliac and superficial cervical lymph nodes of adults slaughtered infected cows.

Antigens for serological test (**Cheville *et. al.,* 1993)**:

Antigen for Rose Bengal Plate test (**Davis, 1971)** : *Br. abortus* antigen for Rose Bengal plate test obtained from Bio – Meriuex Lab reagem and product, France.

Antigen for Buffered Acidified plate agglutination test: *Br. Abortus* antigen for buffered acidified plate test obtained from Veterinary serum and Vaccine Research Institute Abbassiya, Cairo, 11517 , Egypt .

Antigen for complement fixation test **(Alton *et al.,* 1975a)**.

Smooth antigens for TAT and MET Chaves: Standard *Br. abortus* strain agglutination concentrates was supplied from the Veterinary Serum and Vaccine Research Institute, Abbassiya, Cairo, Egypt.

Rough antigen for TAT and MET: A lyophilized *Br. abortus* strain RB^{51} antigen locally prepared in the Department of Brucella, Animal Health Research Institute, Dokki, Giza, according to the technique described by **Stevens *et al.* (1994)**.

Lipopolysaccharide (rough and smooth antigen) : prepared to be used in indirect ELISA. It was prepared in the Department of Brucella, Animal Health Research Institute, Dokki, Giza according to **Alton *et al.,* (1988)**.

Media:

Bacto - brucella agar: was supplied from Difco Laboratories, Detroit, Michigan, USA.

Tryptose agar medium: Trypotse agar medium was obtained from Difco Laboratoories, Detroit, Michigan, USA.

Dyes media: Used for typing of brucella iso-

lates and consists of trypticase soya agar with final dilutions of 1/25.000, 1/50.000 and 1/10.000 of thionin (Difco Laboratories, Detroit, Michigan, USA) as well as basic fuchsin finally diluted to 1/25.000 and 1/50.000 (Sigma Chemical Co. St. Louis Missory , USA).

Antisera:

Monospecific antisera against A, M. & R brucella antigens were obtained from welcome Laboratories, Beckengham, England.

Brucella phages:

Tbilisi (Tb) Phage was offered by the Central Veterinary Laboratory, Weybridge, England.

Reagents:

Bruceller gene was obtained from syn Biotics Europe, Lyon – France and was used for brucelline test.

Anti-bovine IgG conjugated with horsereddish peroxidase was obtained from Syn Biotics Europe, Lyon – France.

Substrate ABTS was obtained from Syn Biotics Europe, Lyon – France.

Methods.

Field and epidemiological observations of tested cows:

Cows of different ages, heifers, pregnant and delivered cows were examined for abortion and breeding troubles including, retained placenta with difficult birth, endometritis and repeat breeder . Data regarding beginning of these troubles were also recorded.

3. Collection of blood samples **(Alton *et al.*, 1988)**.

Blood samples were collected for isolation and identification of bacteria.

4 . Serological according to tests of **Hess (1953)** and **Lambert and Amerault (1962)**.

A – Rose Bengal Plate test (RBPT): The test was done according to **Morgan *et al.*, (1969)** and **Alton *et al.* (1988)**.

B – Buffered Acidified plate antigen test (BAPAT): The test was done according to Angus and Barton, 1984.

C – Tube agglutination test (TAT): The test was done according to the method adopted by the central veterinary laboratory (C.V.L.), Weybride, England as described by **Alton *et al.* (1988)**.

D – Complement Fixation test (CFT): The test was done according to **Alton *et al.*, (1975b)**.

E – Enzyme Linked Immunosorbent Assay (ELISA): The test was done according to **Chand *et al.* (1988)**.

Bacteriological Analysis:

Bacteriological culture specimen were evaluated, this include retropharengeal, supramammary, lymph nodes obtained from animals that were positive sero-test method described by Alton *et al.,* 1988. Biochemical tests, dye sensitivity, exposure to monospecific antisera, susceptibility to antibiotics and lysis by phages were performed on colonies with characteristics typical of genus brucells.

5 – Molecular characterization of brucella species isolated from dairy cows: -

The study was conducted on heifers, pregnant and delivered cows. Blood samples were taken according to the breeding records for comparison among them.

Bacterial strains: the bacterial strains used in this study were isolated by conventional methods from milk samples, aborted fetus, and placenta (J-M. verger, Institute National de la Recherche Agronomique, Nouzilly, France. Strain biotyping was performed by standard methods.

PCR of Bacterial DNA : -

Extraction of DNA was carried out according to **Donis *et al.*, (1986)**.

PCR and oligonucleotide primers: the brucella *Omp* 2 gene was used as target DNA. The forward primer (p1 { 5' TGGAGGTCAGAAATGAAC 3'}) and reverse primer (p2 { 3' GAGTGCGAAACGAGCGC 5' }) of an *Omp* 2 gene segment were obtained from National Bioscience, Inc., Plymouth, Minn.

PCR amplification and digestion of bacterial DNA were performed by the method of **Mullis and Faloona (1987)**.

Results

Immunological tests

The conventional serological tests including Rose Bengal test (RBT), Wright 's sero-agglutination test (SAT) and 2-mercapto-ethanol test (2ME) were performed on sera

samples according to standard techniques **(Altone *et al.*, 1988)**.

Clinical observations and epidemiological studies for brucellosis in investigated farms : A number of 4772 cows (lactating, non lactating and heifers) distributed in two farms in Sharkia province with a history of brucellosis were examined serologically by using milk ring test (MRT), buffered acidified plate antigen test (BAPAT), rosebengal plate antigen test (RBPT), tube agglutination test (TAT), enzyme linked immunosorbent assay (ELISA) as well

Table (1). The incidence of brucellosis in cows in private farms in Sharkia province during the period from 2008 - 2011.

Years	No of Animals	BAPAT		RBPT		TAT		CFT		ELISA	
		R	R %	R	R %	R	R %	R	R %	R	R %
2008	645	16	2.48	16	2.48	16	2.48	18	2.8	18	2.8
2009	588	43	7.31	43	7.31	43	7.31	43	7.31	46	7.8
2010	702	38	5.41	38	5.41	38	5.41	40	5.7	40	5.7
2011	867	18	2.07	18	2.07	20	2.3	20	2.3	20	2.3
Total	2802	115	4.10	115	4.10	117	4.18	121	4.32	124	4.42

Table (2). The incidence of brucellosis in cows individually distributed in different districts of Sharkia province during the period from 2008 - 2011.

Years	No of Animals	BAPAT		RBPT		TAT		CFT		ELISA	
		R	R %	R	R %	R	R %	R	R %	R	R %
2008	526	36	6.84	36	6.84	39	7.4	39	7.4	39	7.4
2009	480	44	9.17	44	9.17	45	9.38	46	9.6	46	9.6
2010	512	50	9.77	50	9.77	53	10.4	53	10.4	53	10.4
2011	452	37	8.19	37	8.19	37	8.19	38	8.4	38	8.4
Total	1970	167	8.48	167	8.48	174	8.83	176	8.9	176	8.9

Table (3). Results of bacteriological isolation of different samples randomly collected from serologically positive infected animals with brucellosis (Reactors) in Sharkia province during the period from 2008 - 2011.

Source of animals	Total No. of Samples	Milk Samples		Lymph nodes tissue specimen	
		(-)ve	(+)ve	(-)ve	(+)ve
Private farms	25	23	2	19	6
Individual animals	25	21	4	20	5
Total	50	44	6	39	11

Table (4). The percentages of abortion rates in private farms distributed in different districts in Sharkia province.

Years	No. of ♀ samples	The number of aborted fetuses	Percentages of abortion %
2008	1030	16	1.55
2009	842	22	2.61
2010	538	17	3.16
2011	392	12	3.06
Total	2802	67	2.39

Table (5). The percentages of abortion rates from individual investigated animals distributed in different districts in Sharkia province.

Years	No. of ♀	The number of aborted fetuses	Percentages of abortion %
2008	654	15	2.29
2009	496	7	1.41
2010	502	13	2.59
2011	318	9	2.83
Total	1970	44	2.23

Table (6). The percentages of cows suffering from brucellosis associated with some breeding troubles from both private farms and individual animals (No of investigated Cows = 50).

Breeding abnormalities	No of investigated cows	Private Farms		Individual animals	
		No of infected cows	%	No of infected cows	%
Retained placenta	150	5	3.3	4	2.7
Difficult birth	150	7	4.7	6	4
Ret. & Diff. birth	150	3	2	2	1.3
Endometeritis	150	4	2.7	3	2
Repeat breeder	150	0	0	1	0.7

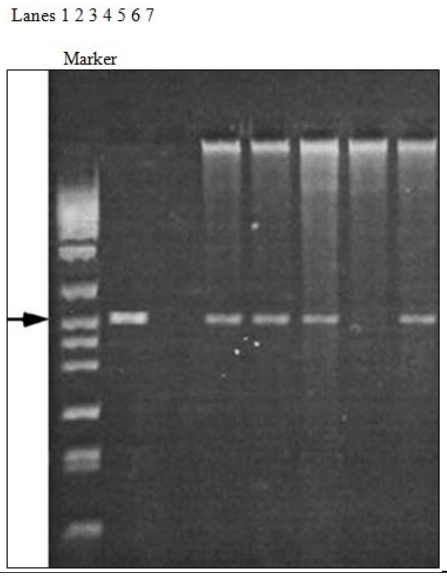

Fig. 1 Agarose gel electrophoresis and ethidium bromide staining. Lane
M standard DNA marker, lane 1 positive control, lane 2 negative control,
Lanes 3, 4,5 and 7 positive blood sample DNA PCR; lane 6 negative
Blood sample for bacterial DNA PCR. The 720-bp PCR Product is indicated.

Discussion

In Egypt brucellosis is a zoonotic with a world-wide distribution , the disease is endemic in the Middle east **(Soliman, 2006)**. In Central and South America brucellosis represents a significant public health issue and its incidence might reach more than 200 cases per 100000 population **(Corbel, 1997)**. Due to misdiagnosis and underreporting though, its true incidence remains unknown and might extend to 25 times higher than the official one **(Taylor and Francis, 2004)**.

The ultimate goals of vaccination are to control disease and reduce or eliminate transmission from reservoir species. To accomplish these goals in ruminants using *Brucella* vaccines, the development of more efficacious vaccination mechanisms are needed to enhance vaccine efficacy.

Tables (1) and (2) shows the incidence of brucellosis in cows (heifers, pregnant and delivered) in some private farms and individual animals; the percentages of reactors serologically were 4.42 % and 8.9 % for cows in private farms and individually collected samples respectively; this agreed with **(Eyles *et al.,* 2001, Sun *et al.,* 2003** and **Kadry *et al.,* 2004)** who observed that vaccine used serves to modify the uptake and processing of antigen. Thus percentage of brucellosis in private farms (4.42 %) was lower than that in individual randomly cows in different districts of Sharkia governorate (8.9 %).

Also, **Kahl-McDonagh and Ficht (2006)** suggested that prolonged persistence of the vaccinal strain in the host was needed for the development of suitable anti-*Brucella* immunity.

Table (3) shows bacteriological isolation of *Brucella* from milk samples by (8 % and 19 %) and from lymph nodes specimen (25 % and 31.6 %) of serologically positive animals of private farms and individual animals respectively; this agrees with **Alton *et al.* (1988)** and **Kadry *et al.* (2004)** who isolated *Brucella* species from milk samples and lymph nodes.

Table (4) shows the percentages of abortion percentages of pregnant cows suffering from brucellosis in some private farms in Sharkia province; the which were 1.55 %, 2.61 %, 3.16% % and 3.06 % among years 2008, 2009, 2010 and 2011 respectively and table (5) shows the percentages of abortion of pregnant cows suffering from brucellosis collected from individual animals in Sharkia province; the percentages of abortions were 2.29 %, 1.41 %, 2.59 % and 2.83 % for yeas 2008, 2009, 2010 and 2011, respectively.

The previous results were lower than those reported by **Salem *et al.,* (1987)**, (16.1 %), **Hamdy (1989)** (37.4 %) and **Montasser *et al.* (2002),** (26 %). Similar results were obtained by Kadry *et al*., (2004) who stated that the prevalence of brucellosis among cattle, buffaloes, sheep and goats were 1.17 %, 0.6 %, 2.2 % and 0.7 % respectively.

Table (6) shows breeding troubles of investigated animals as Retained placenta, Difficult birth, Retained placenta and difficult birth, Endometritis and Repeat breeder in infection with brucella in random investigated animals were 2.7%, 4%, 1.3%, 2% and 0.7 % respectively. From this data we concluded that breeding troubles increase the susceptibility to infection with brucella which agrees with most researchers in brucella as **Alton *et al.,* (1988)** and **Radostis, *et al.,* (2007)** who proved that breeding troubles and poor feeding increase infection with brucella.

The PCR technique has increasingly been used as a supplementary method in *Brucella* diagnosis **(Leal-Klevezes *et. al.,* 1999)**, recently a molecular biotyping approach has been proposed on the basis of restriction endonuclease polymorphism in the genes encoding the major outer proteins of *Brucella* membrane. The Omp2 gene exists as a locus of two nearly homologous repeated copies that differ slightly among *Brucella* species and biotypes **(Ficht *et al.,* 1988)**.

We used previous information to design specific primers that amplify a 720 bp fragment (Fig. 1) lanes 3, 4 and 5 shows the positive samples taken from first farm after vaccination with RB51 vaccine whereas only lane 7 was positive samples collected from second farms. Lanes 6 and 8 were negative for PCR against

Brucella species. **Fichet *et al.*, (1990)** and **Gholam and Masood (2009)** who used P.C.R for amplification of the 720-bp sequence of immunogenic outer membrane protein of gene specific to *Br. melitensis.*

We assumed that the sensitivity of the test would be doubled by selecting duplicated DNA sequences of two genes. Because of the existing *Pst* I site polymorphism between *Br. melitensis* and *Br. Abortus*, thus the researchers suggest that the test is specific for distinguishing between 2 species, which agrees with **Bogdanovich *et al.* (2004)** who used a real time genus specific 5' nuclease PCR assay for amplification of genus *Brucella* and **Gholam and Masood (2009)**.

It could be concluded that much efforts should be focused on the problem of the disease in cattle as they played a role in disease transmission to eliminate it and reduce its prevalence of the disease among cattle.

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مدي تواجد مرض الإجهاض المعدي بين الماشية في محافظة الشرقية

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الملخص العربى

تمت هذه الدراسة علي عدد 4772 من الأبقار في مختلف مناطق محافظة الشرقية (البكر و العشار والتي سبق ان حملت وولدت قبل ذلك) وهي موزعة كالتالي (2802 من المزارع الخاصة , 1970 من الأبقار المنتشرة عشوائيا مع الفلاحين) و قد تم تشخيص الإصابة بالمرض بإستخدام الأختبارات السيرولوجية المختلفة.

وأوضحت هذه الدراسة ان نسبة الإصابة بمرض الإجهاض المعدي كانت 4.42 % , 8.9 % لكل من العينات المأخوذة من المزارع الخاصة و الأبقار المنتشرة عشوائيا بمختلف مناطق محافظة الشرقية علي الترتيب خلال الفترة من 2008 – 2011 م.

وكذلك تم عزل الميكروب علي أوساط غذائية مناسبة من عينات الألبان و أنسجة الغدد الليمفاوية للأبقار المصابة و تم تصنيف المعزولات كيميائيا والتعرف عليه بإستخدام تفاعل البلمرة المتسلسل المتعدد بإستخدام بوادئ جينات خاصة بالبكتريا المعزولة.

وتخلص هذه الدراسة إلي التعرف علي أهمية الماشية في نقل مرض الإجهاض المعدي و إلقاء الضوء علي ضرورة عمل دراسات متقدمة لتقليل الإصابة بهذا المرض و التخلص منه.