



## Genetic Characterization of *Brucella* Species in Some Dairy Farms of the Nile Delta, Egypt

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### Abstract

**B**RUCCELLOSIS, as a highly zoonotic disease, has been endemic in Egypt's Nile Delta since 1939 and affects cattle, buffaloes, sheep, and goats, with severe economic losses, and continues to threaten livestock-based communities. This research investigated the seroprevalence of brucellosis in the organized dairy farms in different governorates of Egypt's Nile Delta. Milk Ring Test (MRT) was a screening test for the bulk milk tank, positive farms were investigated serologically using Rose Bengal Test (RBT) and Buffered Acidified Plate Antigen Test (BAPAT). Milk Ring Test (MRT) screening diagnosed brucellosis on 21 of 65 farms (32.31%), the positive farms being dispersed as follows: 40% in Damietta, 25% in Sharqia, and 30% in Behira. Individual animal serological tests confirmed infection levels of 10.7% in Damietta, 22.9% in Sharqia, and 16.4% in Behira with both RBT and BAPBT. *Brucella* organisms were cultured from 10 farms (47.6% of the seropositive farms). Molecular typing by Abortus, Melitensis, Ovis and Suis Polymerase Chain Reaction (AMOS-PCR) confirmed nine isolates as *Brucella melitensis* biovar 3 and one as *Brucella abortus* biovar 1. These findings highlight the diagnostic challenge of brucellosis in endemic settings, where there is no single definitive test. The prevalence of *B. melitensis* biovar 3 in Egypt's Delta region is fostered by mixed farming and poor hygiene and demands concerted control efforts using serological and molecular diagnostics, test and slaughter policies, and vaccination. Consumption of raw milk and dairy products from infected animals remains a key transmission mode, emphasizing the need for strict food safety policies to protect public health.

**Keywords:** AMOS-PCR, *Brucella melitensis*, Brucellosis, Dairy cattle, Seroprevalence.

### Introduction

*Brucella* bacteria is gram-negative facultative intracellular pathogens for both human beings and other animals, causing an infectious illness widely embraced by the Food and Agriculture Organization (FAO) and World Health Organization (WHO) as among the most widespread zoonotic illnesses with considerable problems based on its impact on animal productivity [1]. The primary symptom that is noticeable for *B. abortus* infection is infertility, which may cause abortion as well as the birth of a weak fetus that can keep infecting other animals [2].

Conservatives estimate that the disease infects more than 300 million of the 1.4 billion world cattle [3]. *Brucellae* are flushed from most infected animals via milk, vaginal secretions, urine, amniotic fluid, fetal membranes, and semen. These bacteria infiltrate the pregnant uterus, fetus, and placenta and cause abortion in the later stages of pregnancy, mainly the last third of the trimester [4]. While these infections are usually permanent, most abortions in infected cows are single occurrences, and the animals are carriers of infection until the next calving [5].

The human transmission may be through several routes, including consuming raw or undercooked

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meat, raw milk, and milk by-products [6]. The bacteria may also gain entry through skin wounds, mucous membranes, or inhalation and hence come into direct contact with contaminated animal tissues or fluids, which could be risky. Tasks such as handling carcasses and helping give birth to animals may raise the risk of coming into contact with infectious fluids and tissues [7].

Diagnosis of Brucellosis depends heavily on the isolation of the causative microorganism, yet still the most crucial step in disease elimination [8]. Brucellosis can be diagnosed using serological tests for the detection of the presence of *Brucella* antibodies [9]. Diagnosis of *Brucella* by serology involves the use of Rose Bengal Test (RBT) and Buffered Acidified Plate Antigen Test (BAPAT) on serum [10], while Milk Ring Test (MRT) and Indirect Enzyme Linked Immunosorbent Assay (i.ELISA) is a reliable method for detecting *Brucella*-specific antibodies in milk [11] and all before the Polymerase Chain Reaction [12].

Abortus, Melitensis, Ovis and Suis Polymerase Chain Reaction (AMOS PCR) technique has been demonstrated to be a very effective method for the sensitive, rapid, and precise detection of *Brucella* species. [13]. In Egypt, though, only a few laboratories can successfully isolate *Brucella* [14]. Routine methods for *Brucella* isolation from clinical and non-clinical samples use enriched media, such as Farrell's, Castaneda's, and modified Thayer–Martin media [15]. Despite the control program, the disease is still endemic among livestock and humans [14].

Effective eradication of brucellosis requires strict prevention and control efforts that include herd detection of infected herds, animal movement control, and implementation of quarantine with penalties for any unauthorized movement. Despite vaccination efforts, quarantine enforcement, and paid slaughtering programs, the disease continues to exist in the Delta area due to unregulated livestock movement and inadequate veterinary services [16]. Outbreaks in both animals and humans must be tracked, contained, and their sources pinpointed using epidemiological and molecular techniques [17]. Furthermore, strategies to curb brucellosis transmission through milk and dairy products are aimed at the application of severe heat treatment processes before use and the intensification of protective action at the dairy chain of supply [18].

This study aimed to assess the prevalence of *Brucella* species among organized dairy farms by examining raw milk and serum samples to identify the prevalent serotypes in the Nile Delta.

## **Material and Methods**

### *Experimental design*

Sixty-five dairy cattle organized farms in the Damietta (25 farms), Sharqia (20 farms), and Behira

(20 farms) governorates, provided Bulk Milk Tank (BMT) samples for initial screening using Milk Ring Test (MRT). Adult dairy cows (3-5 years old) were used for milk and serum sampling. Twenty-one farms were positive for Milk Ring Test (MRT) and were subsequently tested with serum samples by both Rose Bengal Test (RBT) and Buffered Acidified Plate Antigen Test (BAPAT). Clinical data, including farm management, herd size, age of animals, history of abortion as in Figure (2), and location of the farm, were collected by questionnaires from the farm owners, veterinarians, and farm laborers.

### *Sample collection*

#### *Milk samples*

Milk samples were collected from 65 bulk milk tanks of private dairy farms following the method of [8]. The milk samples were collected from the bulk cooling tanks on each farm. The milk in each tank was mixed continuously until the top of the tank was opened before sampling. Sterile single-use plastic pipettes were used to aseptically pipette the milk directly from the tank. The samples were all treated under strict aseptic conditions as they were intended for the identification of *Brucella*.

#### *Blood Samples*

Serum samples were collected from Damietta (1499), Sharqia (929), and Behira (736) governorates as per the method of [19]. Blood was drawn from the jugular vein into sterilized tubes free from anticoagulant and left to clot at room temperature for one hour before refrigeration overnight. After centrifugation at 3000 rpm for five minutes, the clear sera were siphoned off and stored at -20°C before being shipped to the laboratory for analysis.

### *Serological analysis*

Milk samples were tested with Milk Ring Test (MRT), provided by AHVLA, New Haw, Addlestone, Surrey KT15 3NB, UK. Milk Ring Test (MRT) was carried out according to the OIE Manual (20). All serum samples were screened using both Rose Bengal Test (RBT) and Buffered Acidified Plate Antigen Test (BAPAT) as per the guidelines in the OIE Manual. The antigens for these tests were sourced from the Veterinary Serum and Vaccine Research Institute in Abbassia, Cairo, Egypt.

### *Bacteriological isolation and identification [8]*

Direct bacterial culture was subsequently done on the brucellosis-positive milk samples of dairy farms. After centrifugation at 6000 g for 10 minutes, the cream layer was aspirated, and the sediment was admixed with it. Then it was inoculated onto tryptic soya agar media plates to incubate. These were incubated at 37°C under conditions with and without the addition of 5-10% carbon dioxide. Growth was observed after 3-5 days, followed by 8-10 days of

examinations, and thereafter every day for 15 days. Suspected colonies were also viewed microscopically and tested using *Brucella*-positive and *Brucella*-negative sera.

#### *Molecular examinations*

DNA was extracted from obtained isolates using the QIAamp DNA Mini kit (Qiagen, Germany) with minor modifications. A 200 µl sample was lysed with buffer and proteinase K, incubated, then mixed with ethanol, washed, centrifuged, and DNA was eluted with 100 µl of elution buffer.

To amplify by PCR, a 25 µl reaction was established with Emerald Amp Max PCR Master Mix, species-specific primers against the insertion sequence IS711 downstream of the *bcs*p31 gene, 1 µl of each of the primers (20 pmol), 5 µl of template DNA, and 5.5 µl of water in an Applied Biosystems 2720 thermal cycler.

AMOS-PCR included initial denaturation at 94°C for 5 min, followed by 35 cycles (94°C for 30 sec, 55°C for 40 sec, 72°C for 45 sec), and a final extension at 72°C for 10 min. The PCR products were analyzed on a 1% agarose gel in 1x TBE buffer (5V/cm) using a 100 bp DNA ladder, with gels photographed for data analysis (21).

### **Results**

Bulk Milk Tank (BMT) samples were collected from organized dairy cattle farms from various governorates of Egypt's Nile Delta region for conducting an initial screening for brucellosis by the Milk Ring Test (MRT), as outlined in Table 1. The positive MRT farms were again investigated, where cattle's serum samples were tested using both RBT and BAPAT. Concretely, all confirmed positive samples had concordant results in both RBT and BAPAT, as indicated in Tables (2), (3), and (4).

### **Discussion**

Brucellosis is a zoonotic infection that results in great economic loss due to reproductive abnormalities in animals like abortions and infertility [22]. It is an occupational risk to farmworkers, slaughterhouse employees, and veterinarians [5]. Brucellosis is endemic in Egypt and the Mediterranean area, where diagnostic accuracy continues to pose a challenge regardless of control strategies. The true prevalence and incidence are unknown, and predictive tests of recovery following treatment are unreliable. The assessment of the true epidemiological burden and serum antibody titres among urban and rural populations is important in evaluating treatment schemes to reduce relapse rates [23].

Consequently, this study aimed to examine the prevalence of brucellosis in dairy cattle on organized farms in the Delta region utilizing widely accepted diagnostic methods, including serological,

bacteriological, and molecular techniques, as well as to identify the specific *Brucella* species present in the research area. The findings affirmed the continued existence of endemic brucellosis in the region, with a variable infection rate within the studied governorates and farms.

After an increase in the cases of abortion, bulk milk tank (BMT) samples were collected from 65 dairy farms located in Damietta, Sharqia, and Behira governorates. Brucellosis screening by MRT identified 21 positive farms (32.31%), which were further confirmed by both BAPAT and RBT. The positivity was 40% in Damietta, 30% in Behira, and 25% in Sharqia, as indicated in Table 1.

These results point to the ongoing risk of *Brucella* infection in dairy herds and support previous reports on the endemicity of brucellosis in Egyptian livestock [14]. The differences in MRT positivity within the various governorates may reflect differences in herd management, biosecurity, or animal movement practices compared to the other governorates. The lower positive results obtained using the MRT may be due to its lower sensitivity, particularly in the detection of antibodies in milk samples with low antibody concentration or when the fat aggregation interferes with the test accuracy [21]. This explanation was supported by [13], who mentioned that the investigation into brucellosis prevalence revealed that 48 samples (7.8%) tested positive via BAPAT, while RBPT identified 44 samples (7.2%), and MRT detected 41 samples (6.7%).

Scientists universally concur that no one serological test can correctly diagnose positive cases in animals. The RBT and BAPAT continue to be the most prevalent rapid screening tests used for the identification of positive animals [13]. Furthermore, the seroprevalence of brucellosis using both RBT and BABAT was 161(10.74%) in Damietta, Table 2, 213(22.93%) in Sharqia, Table 3, and 121(16.44%) in Behira, Table 4. Based on a detailed inspection of serological test results and clinical brucellosis signs observed, such as abortions, endometritis, and placental retention, a quick decision was made to slaughter infected animals to avoid further infections and abortion cases. For this reason, further confirmatory tests were not pursued. In endemic regions, particularly where there is severe manifestation, removal of animals with identical positive results in two serological tests is warranted [10].

The endemic level of brucellosis in certain areas poses challenges linked to the complexities of serological testing, especially the likelihood of false-positive results, which represent major hurdles in accurately diagnosing and preventing the disease [24]. There is no agreement on the best serodiagnostic tests for brucellosis due to the absence

of a definitive test for comparison with other laboratory methods. Alternative diagnostic approaches include cultures and PCR for detecting pathogen nucleic acids. Serological tests are often assessed by comparing their results with those from other serological methods, used either individually or together. This lack of consensus is partly due to the disease's progression in either acute or chronic stages [25].

For the diagnosis of brucellosis, bacterial isolation necessitates a properly equipped laboratory that meets biosafety level 3 standards, along with highly trained personnel capable of managing these microorganisms [26]. *Brucella* colonies were successfully isolated from various bulk milk samples taken from 21 seropositive cattle farms. The failure to isolate bacteria in some serologically positive cases, which accounted for 47.62% (10 out of 21) as in Table 1, was linked to a low viable *Brucella* load in the bulk milk samples or due to bacterial contamination, however, the complex growth requirements of *Brucella* spp. also contributed to this issue [27].

The characterization of the ten *Brucella* isolates included a combination of growth characteristics, which served as the definitive identifiers for *B. melitensis* biovar 3 and *B. abortus* biovar 1, Table 1. These results align with earlier studies conducted by [28], who isolated *B. melitensis* and *B. abortus* from diverse animal species and different governorates in Egypt.

The molecular method for bacterial characterization is five times faster than traditional bacterial isolation methods. This approach mitigates the challenges associated with biohazard management, lowering both contamination risks and diagnostic costs [29].

Ten *Brucella* strains were identified in milk samples cultures in this study. AMOS-PCR identified

nine as *B. melitensis* biovar 3 and one as *B. abortus* biovar 1, Figure (1). Previous reports also stated that *B. melitensis* biovar 3 is the prevailing strain in Egypt [4].

### **Conclusion**

Brucellosis remains a significant public health and economic concern in Egypt, particularly in the Nile Delta, with *Brucella melitensis* biovar 3 endemic in cattle. Human infection is largely due to the consumption of raw milk products and occupational exposure. The persistence of the disease is maintained through inadequate hygiene, mixed livestock management, and environmental contamination.

Due to the limitations of diagnosis in endemic areas, an integrated approach is necessary, combining serological and molecular diagnosis, screening on a regular basis, culling of infected animals, and vaccination. Preventive measures, including pasteurization of milk, proper cooking of meat, wearing protective coverings, proper handling of vaccines, and public awareness, are significant in controlling transmission and guarding both animal and human health.

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### **Declaration of Conflict of Interest**

The authors declare that there is no conflict of interest.

### **Ethical of approval**

This study follows the ethics guidelines of the Faculty of Veterinary Medicine, Mansoura university, Egypt.

**TABLE 1. Prevalence of brucellosis in dairy cattle on organized farms at different governorates depending on MRT:**

Governorates	No. of farms	MRT +Ve Cases (%)	<i>Brucella</i> isolates	<i>Brucella</i> spp. by AMOS-PCR
Damietta	25	10 (40%)	6 (60%)	(1) <i>B. abortus</i> bv1 (5) <i>B. melitensis</i> bv3
Sharkia	20	5 (25%)	3 (60%)	<i>B. melitensis</i> bv3
Behira	20	6 (30%)	1(16.67%)	<i>B. melitensis</i> bv3
Total	65	21(32.31%)	10(47.61%)	

**TABLE 2. Prevalence of brucellosis in different cattle herds at Damietta governorate depending on RBT and BAPAT:**

Herd	Animals No.	+Ve Cases (%)
Herd 1	120	26(21.7%)
Herd 2	150	8(5.3%)
Herd 3	120	35(29.2%)
Herd 4	80	4(5%)
Herd 5	150	8(5.3%)
Herd 6	169	44(26.03%)
Herd 7	140	4(2.9%)
Herd 8	252	13(5.2%)

<b>Herd 9</b>	168	13(7.7%)
<b>Herd 10</b>	150	6(4%)
<b>Total</b>	1499	161(10.74%)

**TABLE 3. Prevalence of brucellosis in cattle herd at Sharkia governorate depending on RBT and BAPAT:**

<b>Herd</b>	<b>Animals No.</b>	<b>+Ve (%)</b>
<b>Herd 1</b>	123	15 (12.2%)
<b>Herd 2</b>	110	5(4.54%)
<b>Herd 3</b>	437	133(30.43%)
<b>Herd 4</b>	189	39(20.63%)
<b>Herd 5</b>	70	21(30%)
<b>Total</b>	929	213(22.93%)

**TABLE 4. Prevalence of brucellosis in cattle herd at Behira governorate depending on RBT and BAPAT:**

<b>Herd</b>	<b>Animals No.</b>	<b>+Ve (%)</b>
Herd 1	90	2(2.22%)
Herd 2	90	4(4.44%)
Herd 3	121	52(42.9%)
Herd 4	15	15(100%)
Herd 5	240	40(16.7%)
Herd 6	180	8(4.44%)
Total	736	121(16.44%)

**Fig. 1. Molecular identification of *Brucella* isolates recovered from milk samples:****Fig. 1. Differentiation of *Brucella* species by AMOS-PCR.** Lane 1; DNA ladder, L2; control negative; lane 3, *B. abortus* reference strain 544; lane 4, *B. melitensis* reference strain Ether; isolates (1-3), *B. melitensis* field isolates displayed bands at 731 bp; isolate 4, *B. abortus* field isolates exhibit band at 498 bp; isolates (5-10), *B. melitensis* field isolates (731 bp).**Fig. 2. Aborted Holstein fetus at 7<sup>th</sup> month of gestation in Damietta Governorate due to brucellosis:**

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## التوصيف الجيني لأنواع البروسيلاء في بعض مزارع الألبان بدلتا النيل، مصر

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<sup>3</sup> كلية الطب البيطري، جامعة بدر بالقاهرة (BUC)، ص.ب 1182، مصر.

### الملخص

إن داء البروسيلاء، يعد من الأمراض المشتركة بين الحيوان والإنسان، فلا يزال موجودًا بشكل مستوطن في دلتا النيل في مصر منذ عام 1939، ويؤثر على الماشية مثل الأبقار والجاموس والأغنام والماعز، مما يسبب خسائر اقتصادية جسيمة كما يهدد البيئة الزراعية والتي تعتمد اعتمادًا كليًا على تربية الماشية. تهدف هذه الدراسة إلى التركيز على معدل انتشار البروسيلاء في محافظات مختلفة من دلتا النيل في مصر عن طريق إجراء بعض الاختبارات السيرولوجية على بعض من مزارع الألبان. حيث تم استخدام اختبار اللب الحلقى كاختبار فحص أولي لخزانات الحليب بالمزارع؛ وتبين انتشار معدل الإصابة بالبروسيلاء في 21 مزرعة من أصل 65 (32.31%)، حيث توزعت الإصابات الإيجابية على النحو التالي: 40% في دمياط، و25% في الشرقية، و30% في البحيرة. وقد أكدت الاختبارات السيرولوجية الفردية للحيوانات المصابة معدلات الإصابة على النحو التالي: 10.74% في دمياط، و22.93% في الشرقية، و16.44% في البحيرة باستخدام اختبار الانتيجين الشريحي المحمض واختبار الروزبنجال. وقد تم عزل البروسيلاء من 10 مزارع (47.61% من المزارع التي أظهرت نتائج إيجابية سيرولوجياً). وأظهرت النتائج أن 9 معزولات من البروسيلاء *Brucella melitensis* biovar 3، ومعزولة واحدة من نوع *Brucella abortus* biovar 1 كما أكدت تقنية AMOS PCR ذلك. تسلط هذه النتائج الضوء على التحديات التشخيصية لمرض البروسيلاء في البيئات المستوطنة للمرض، حيث لا يمكن الاعتماد على اختبار سيرولوجي واحد في تشخيص البروسيلاء. كما أثبتت أن *B. melitensis* biovar 3 هي الأكثر شيوعاً في منطقة دلتا مصر ويرجع ذلك إلى عدة عوامل منها التربية المختلطة وعدم الالتزام بممارسات النظافة الجيدة، مما يتطلب جهود مشتركة للسيطرة باستخدام التشخيصات السيرولوجية والجزيئية، وإيضاً تساعد استراتيجيات الاختبار والذبح في إدارة تفشي المرض و لذلك يجب اتباع الأنظمة الصحية الصارمة بالتوازي مع زيادة وعي المربين بخطورة المرض وأهمية التحصين للحد من انتشاره بين الحيوانات المختلفة. ويظل استهلاك الحليب الخام ومنتجات الألبان من الحيوانات المصابة أسلوب نقل رئيسي، مما يبرز الحاجة إلى سياسات صارمة لسلامة الغذاء لحماية الصحة العامة.