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# Comparable Study of Immunological, Bacteriological, and Molecular Techniques for Detecting Brucellosis in Milk of Reproductively Problematic Cows

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BRUCELLOSIS, a serious animal infectious disease transmitted to human beings, is a major health risk for dairy consumers, especially in developing-countries resulting in dangerous economic and financial problems. Brucellosis diagnosis is critical in order to detect this infection and find a suitable treatment. Accordingly, this study aims to follow up on the accuracy and sensitivity of different diagnostic techniques used in detecting brucellosis in milk (immunological, bacteriological, and molecular techniques). Milk ring test (MRT) and indirect enzyme-linked immunosorbent assay (i-ELISA) are among the used techniques where positive brucellosis was detected in milk samples with total percentages of 34.91% and 33.96%, respectively. The recorded relative-sensitivity for MRT and i-ELISA were 93.55% and 96.77%, respectively, while their relative-specificities were 89.33% and 92%, respectively. i-ELISA was found to show both higher relative sensitivity and specificity than the MRT technique. The percentage of *Brucella* species detected in milk samples via bacteriological culture was 25.5% and all of them were reported to be *B. melitensis* biovar 3. On the other hand, 28.3% of samples were positively detected in both conventional and qPCR samples. The relative-sensitivities were 87.10%, 96.77%, and 96.77% for bacteriological culture, conventional-PCR, and qPCR techniques, respectively. All three techniques reported relative-specificities of 100%. The most predominant species detected in cows' milk suffering from reproductive disorders is *B. melitensis* biovar 3. Both PCR techniques showed the highest relative-sensitivity and specificity in addition to being rapid and low-risk techniques making PCR the best diagnostic technique for brucellosis.

**Keywords:** Milk, Diagnostic techniques, Relative sensitivity, Relative specificity.

## Introduction

Brucellosis, a zoonotic disease, has a notable and serious economic impact on livestock. The most common reason for this infection is Brucella species, which can infect certain animal species. This infection can be transferred to humans when they consume contaminated milk from diseased cows. This contagious disease results in debilitating illness. Brucellosis doesn't only transfer through direct contact, like drinking milk, or between animals during herding and grazing; it also transfers indirectly between farmers, slaughterhouse workers, and veterinarians while dealing with the infected animals' excretions, genital secretions, milk, aerosols, and waste materials [1]. It became a health threat as a result of its rapid transmission to humans from infected animal-derived food. Accurate diagnosis of this

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bacterial disease is a fundamental challenge, as is finding a suitable medication to eradicate it [2]. There are already some traditional techniques used in diagnosis, like the Rose Bengal tube test, serum agglutination test, and enzyme-linked immunosorbent assay (ELISA). A gold-standard method for diagnosis is pathogen isolation, although isolation has zoonotic characteristics due to the intracellular and fastidious nature of Brucella spp. A more accurate method, now gaining higher acceptance than immunological tests, is using PCR techniques for confirming the pathogen isolates [3, 4]. Accordingly, our objective in this paper is to follow up on the accuracy of the widely used diagnostic immunological techniques (MRT and indirect ELISA [i-ELISA]), bacteriological culture, and molecular methods (conventional and qPCR) for Brucella species identification from cows' milk suffering from reproductive disorders. This investigation determined the relative sensitivities, relative specificities, and predictive positive and negative values for effective control strategies of these different diagnosis techniques.

## Material and Methods

One hundred and six milk samples were used in this study, divided into 32 samples from apparent health cows (negative control) (randomly selected without any reproductive disorder history and regular lactation) and 74 samples from cows suffering from reproductive abnormalities. The experimental samples were chosen according to the animals' cases: 22 samples for late aborted animals, 28 samples for cows with retained placentas, and 24 for repeat breeders. All the samples were obtained from various herds in the Giza governorate, which contains six cow herds, each containing approximately 100 cows. Out of them, three dairy herds at different lactation stages (beginning, middle, and last) were chosen for this study.

The Egyptian Network of Research Ethics Committees (ENREC) has approved collecting milk samples non-invasively using the conventional-hand-stripping method. 50 mL of the collected milk sample was sent on ice to the Animal Health Research Institute for examination. The sample was divided into 10 aliquots (5 mL each), subsequently used for immunological, bacteriological, and molecular examinations.

*Immunological examination*

*Milk Ring test (MRT)*

For this test, 30 µ1 MRT antigen was supplied by "The Animal Health Veterinary Laboratories" Agency (AHVLA), DEFRA, UK. Then 1ml of freshly collected milk (within 2 hours of collection), the milk was stored for at least 72 hours at 6 degrees C [5], was mixed well with this antigen, and then incubated for 3 hours at 37 °C. Positive results are those showing purple bands on the top of tested samples [6].

*Indirect Enzyme-Linked Immunosorbent Assay (i-ELISA)*

The i-ELISA kit (SVANOVIR Brucella-Ab C-ELISA, Sweden) was used to detect anti-Brucella antibodies in fresh milk samples. The procedures and both positive and negative controls were applied according to the manufacturer's instructions. The milk cream layer was removed by centrifuging the obtained samples at 5000 rpm for 5 minutes as a preparatory step for detecting antibodies. The "Sample Dilution Buffer" solution was divided across 45 microliters in each well. To the initial wells (A1, A2, B1, B2, C1, C2, D1, and D2), five microliters of the positive control, low positive, negative control, and sample dilution buffer were added in duplicate. The sample serum was divided into five microliters and added to the remaining wells. The wells received forty microliters of mAb-solution added to them. Following a five-minute shake in a plate shaker, the plate was covered and allowed to incubate for a half-hour at room temperature. Following the incubation period, the wells underwent four rounds of washing and rinsing with the PBS-Tween buffer solution. The conjugate solution was applied to 100 microliter wells, and they were then incubated for 0.5 hours at room temperature. The conjugate solution was applied to 100 microliter wells, and they were then incubated for 0.5 hours at ambient temperature. The PBS-Tween buffer solution was used to wash the wells one more. After adding 100 microliters of the substrate solution, the mixture was allowed to sit at ambient temperature for 10 minutes. To stop any more reactions, 50 microliters of the stop solution were added. The microplate photometer operating (ThermoElectron, Finland) at 450 nm wavelength was used to evaluate the results.

*Bacteriological examination*

Milk samples were plated on Brucella agar (HiMedia, India) plates following a 24-hour enrichment in Brucella broth (HiMedia, India) and then incubated at 37 °C in 10% $CO_2$ ($CO_2$ incubator, INCUCELL) and daily examined for colony morphology, Oxidase, urea hydrolysis, H2S production, growth on basic Fuchsin

concentration in serum dextrose agar (20 µg/ml) and agglutination with monospecific sera (A, M, R) in compliance with the OIE [7].

*Molecular examination*

*DNA extraction from milk samples*

Extraction of DNA from the collected milk samples by NZY® gDNA isolation kit that was applied according to the kit manufacturer's instructions. A volume of 1400 µL of each collected milk sample was centrifuged in micro-centrifuge tubes for 5 min at 3000 rpm. The obtained supernatant of cream and milk was discarded and the formed pellet was transferred to new micro-centrifuge tubes (extracted DNA) and reserved at -80 °C.

*Conventional PCR*

A conventional PCR technique was used for amplifying the target gene with molecular weight 223 bp [6] from the extracted DNA using DNA thermal cycler type (Perkin-Elmer model 9600). The chosen forward and reverse primers were B4 and B5, respectively (Table 1). The positive control was (*B. melitensis* Rev1 DNA), while the negative control was set to sterile distilled water (instead of the template) for contamination (to ensure that the reaction mix was not contaminated) and false-positive detection.

*Electrophoresis of PCR product:*

The PCR products of amplified DNA sequences were electrophoresed on 1.5% agarose gel after mixing 5 µL of each product with 1 µL of 6X gel loading dye. The electrophoresis was running for 30 minutes on a voltage of 100 and a UV transilluminator was used to observe the specified bands using a 50bp ladder (BERUS 50bp Ladder).

*DNA amplification in qPCR:*

The qPCR detection kit for Brucella spp. (Primer Design®, JN68G10-21593) was used for amplifying the target sequence from the previously extracted DNA of each milk sample. According to the manufacturer protocol, 10 µl of 2X Precision™ ®master mix was mixed with 5 µL of extracted DNA. Then the volume was completed to 20 µl by primer/probe mix and RNAse/DNAse-free water (1 µl). The first denaturation step was set for 10 min at 95 °C, followed by 40 PCR cycles where denaturation and annealing of each cycle were set to 95 °C for 10 sec. and 60 °C for 1 min (annealing and extension), respectively. The kit's positive and negative controls were used.

*Estimation of Relative Sensitivity, specificity, and estimated false positives of different diagnostic tests:*

Below equations were used to calculate the Relative-Sensitivity, Relative-Specificity, and Estimated false positive values of the 5 applied diagnostic techniques used in detecting brucellosis in milk [9].

NB: Both true positive and true negative results have been confirmed as positive or negative using two or more other methods. False positive means getting a positive result when it's not true, and false negative means getting a negative result when it's not true. These results can happen using different methods or not using methods at all.

## Results

*Results of Immunological examination*

Among the examined milk samples, 37/106 forming 34.9% were positive for brucellosis by MRT, but 36/106 forming 43% were positive by i-ELISA (Fig. 4). Meaning that, MRT showed a bit higher positive values than those detected by i-ELISA. There was no significant difference between the 2 tests.

*Results of Bacteriological Examination*

Our results showed that 27/106 forming 25.5% of the examined milk samples were found to be Brucella spp. that grow on Brucella agar with smooth colonies, were oxidase-positive, and urea hydrolysis-positive. However, all the obtained isolates were identified as *B. melitensis* biovar 3 species ($H_2S$ positive, could grow in basic fuchsin, and were agglutinated with monospecific sera (A and M)) (Fig. 4).

*Results of Molecular examination*

**Conventional PCR**

Only 30 samples out of the 106 examined milk samples (percentage of 28.3%) (Fig. 4) show positive results with conventional (Figures 1 and 2).

qPCR

q-PCR technique detected Brucella in 28.3% of examined milk (Fig. 5).

*Results of the Relative sensitivity, and specificity of different screening tests*

As Fig. 3 shows the calculated MRT relative sensitivity was 93.55% while its relative specificity was 89.33%. The i-ELISA test recorded higher relative sensitivity and specificity than that of MRT.

However, the relative specificities of all three tests bacteriological culture, conventional PCR, and qPCR were 100% with relative sensitivities of 87.10%, 96.77%, and 96.77%, respectively.

## Discussion

Controlling *Brucella* spp. infection accurately is critical to preventing the disease in animals and, by extension, humans. The history of reproductive failures in livestock is typically the basis for clinical diagnosis; however, this is an assumption-based diagnosis that needs to be verified with laboratory procedures. ELISA and MRT are among the immunological checks. These are followed by isolation and molecular biological investigation, such as polymerase chain reaction (PCR) [10].

Immunological tests (MRT and ELISA) are often used to detect *Brucella* species' antibodies in milk [11]. The most commonly used technique for detecting brucellosis in dairy cows' farms is MRT (field technique) [12]. This study showed that there were more positive cases detected using MRT compared to the milk ELISA test. According to the results, 34.9% of the animals tested were detected with brucellosis via MRT, whereas 33.96% were detected positive by i-ELISA (Figure 4). These results support the results of [13] where more positive results were reported using MRT (51%) than those detected by ELISA (50%). Also, according to the [14] study, the MRT testing method showed a 20% positivity rate for the examined milk samples. Meanwhile, milk-ELISA (mELISA) indicated an 18.1% positivity rate. Similarly, we were able to detect more brucellosis in milk samples via MRT with a false-positive percentage of only 10.67%. However, better results were observed utilizing i-ELISA where only 8% were detected as false positive results (Figure 3) confirming [15] previous results. This variation among different serological tests may have more than one reason like the infection status of the animal from which the milk sample was collected, how the samples were transported and stored, and also aspects where the owners did not provide all the information (e.g., vaccination status) [15]. Even if there is a culture-based infection status, there are still a lot of other variables that may affect the results [15]. It is possible that the high number of positive results from MRT testing could be due to various factors such as the presence of colostrum, particularly if the milk was collected towards the end of the lactation cycle [7]. I-ELISA false positive sample results may be due to the detection of *Yersinia enterocolitica* 09 and *Escherichia coli* 0157:H7 lipopolysaccharides (LPS) and also ELISA can detect *Salmonella* spp. and *Pasteurella* spp. antigens which are also the reasons for PCR true-negative responses [16]. *Brucella* spp. is a meticulous bacterium, it needs numerous nutrients to grow in addition to suitable storage and rapid transportation of collected samples in order to contain a significant number of viable bacteria for diagnosis [17]. All the isolated *Brucella* spp. in this study were *B. melitensis* biovar 3 (25.47%) suggesting that it is the most frequent existing species in the milk of reproductive disordered cows. This correlates with [18] findings which also could isolate *B. melitensis* biovar 3 from cows after abortion. *Brucella melitensis* mainly affects sheep and goats. Furthermore, research has shown that when a particular animal species contaminates an environment, other animals of different species brought into the same environment can also pick up the infection [19].

Di Bonaventura et al. [20] reported that the bacteriological culture method is the "gold-standard" in detecting *Brucella* and that may be the reason for Zero reported false-positive samples for this technique in our current study. The noticed lower sensitivity of the culture technique has been previously mentioned [18] and this may be due to the bacteria's fastidious nature as mentioned by [5].

The application of PCR techniques for brucellosis genetic identification is rising in acceptance as a best practice. The low price of the technique with its ability to detect low concentration of DNA includes genus-level PCR approaches [22]. 28.3% of the 106 milk samples examined by conventional PCR were positive. As well the same result was obtained by qPCR PCR (28.3%). The results obtained through this molecular approach surpass those found by ELISA. It has been suggested that milk samples that test positive for ELISA but negative for PCR may be due to due to that antibodies can cross-react with other pathogens but PCR can detect a unique target gene for brucella [23].

For PCR studies to be successful, pure DNA must be extracted from the milk sample as it

contains higher levels of other components than DNA like milk proteins, fats, proteinases, polysaccharides, and ions like $Ca^{2+}$ [24]. We employed an extraction kit to increase the simplicity and ease of the current investigation and DNA was extracted and amplified as mentioned in the methods section. Based on the data acquired and the ability of PCR to detect DNA in false-negative ELISA samples, these methods and appropriate for milk control risk analysis. Table 1 records the correlation between reproductive disorders and brucellosis infection where higher rates of infection were reported in cattle with reproductive problems than in cattle without recorded disturbance. A link between brucellosis infection and infertility retained placenta, and abortion was reported by [23] which confirms our findings.

The findings suggest that cattle that tested positive for antibodies and had no prior medical history of reproductive problems are the main source of infection and failure to control.

Brucellosis infection depends on the type of reproductive disturbance where the highest infection rates were reported in aborted cows (Table 1). The findings suggest that cattle with a history of reproductive disorders are still more prone to contract brucellosis infection, necessitating more efforts and an effective approach to disease control and eradication.

Brucellosis infection occurs through contaminated milk and dairy products in endemic nations, posing an increasing threat to individuals and entire families [26].

In most places, the sale of unpasteurized milk and dairy products has to be tightly restricted to only farms that have been confirmed to be Brucella-free. In addition to that, both farmers and consumers have to be well educated about this disease's nature and transmission via contacting infected animals and their excretions including their milk and dairy products. They have also to disprove their beliefs that pasteurized milk is better than raw ones.

## Conclusion

False positive records have a negative impact on the herd economy. On the other hand, if any of the cattle show false negative results while they are infected this will negatively impact humans and the environment.

In this study and according to the literature, a routine procedure for detecting *Brucella* spp. is the It is obvious that ELISA was a sensitive test and can be used as iimunoservay. Also, it can be used PCR test only for positive cases as a definitive test.

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*Conflicts of interest*

The authors declared no competing interests.

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**TABLE 1. primers sequence used in the conventional PCR.**

| Target gene | Sequence (5′–3′) | Amplicon size (bp) | PCR conditions and cycles | Reference |
|---|---|---|---|---|
| B4 | F- TGG CTC GGT TGC CAA TAT CAA | 223 bp | 94°C for 3 min for to initial denaturation, followed by 40 cycles for, denaturation at 95 °C for 20 sec., annealing for 60 °C for 30 sec. to annealing, and 72 °C for 1 min. to extension. The final extension phase was set for 7 min at 72 °C. | [8] |
| B5 | R- CGC GCT TGC CTT TCA GGT CTG | | | |

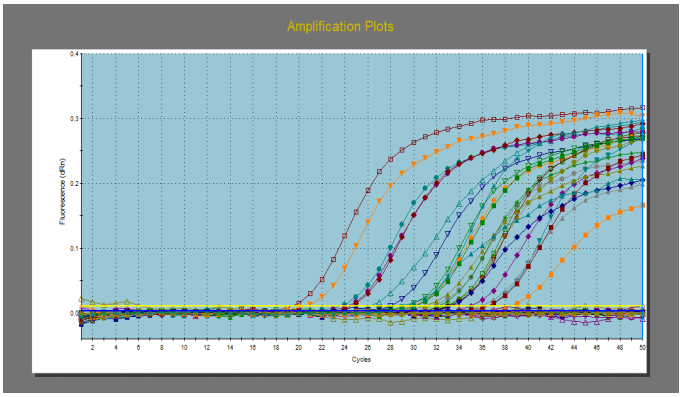


**Fig. 1. Amplification plots for qPCR assay showing amplification curves of some tested samples and one positive control (Brucella template). Curves below the threshold were negative tested samples and one negative control (NCs)**

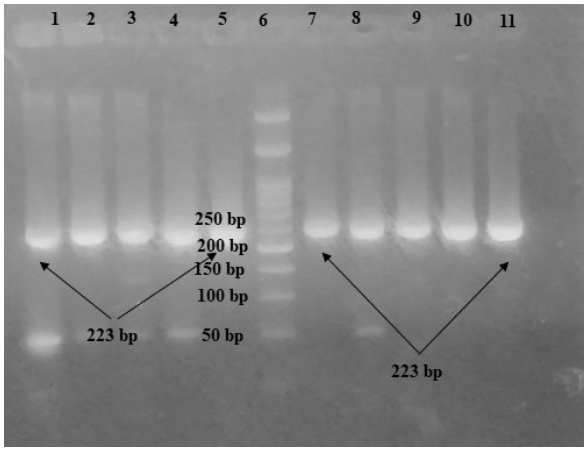


**Fig. 2. Agarose gel electrophoresis PCR products showing positive amplification using PCR with amplification of 223 bp for Brucella spp. gene performed with their specific primer**
**Lane 1-5: positive amplification of 223bp.**
**Lane 6: 50 bp DNA ladder.**
**Lane 7-11: positive amplification of 223 bp.**

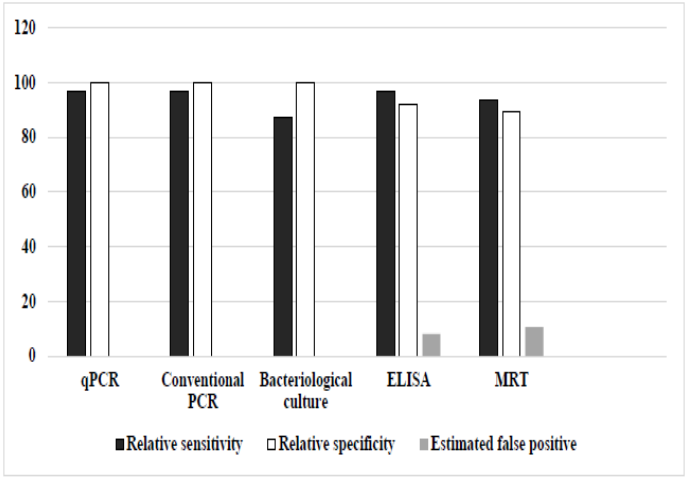


**Fig. 3. Relative sensitivity, Relative specificity, and estimated false-positive percentage of different tests**

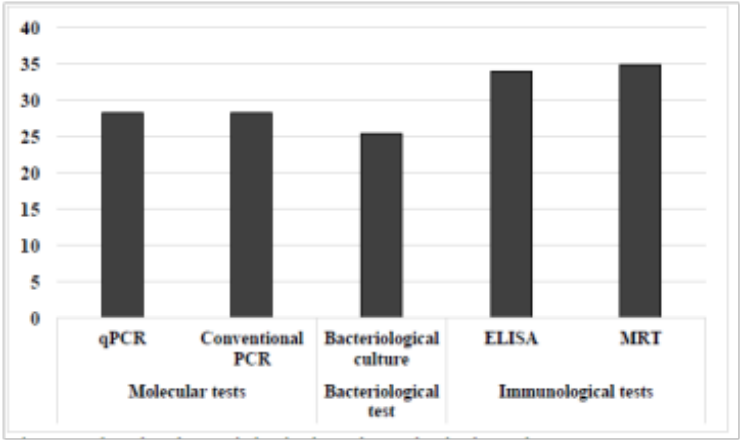


**Fig. 4. Molecular, bacteriological, and immunological results**

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# دراسة مقارنة للتقنيات المناعية والبكتريولوجية والجزيئية للكشف عن داء البروسيلات في حليب الأبقار التي تعاني من مشاكل إنجابية

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يعد داء البروسيلات مرضًا معديًا حيوانيًا خطيرًا ينتقل إلى البشر، ويشكل خطرًا صحيًا كبيرًا على مستهلكي الألبان، خاصة في البلدان النامية مما يؤدي إلى مشاكل اقتصادية ومالية خطيرة. يعد تشخيص داء البروسيلات أمرًا بالغ الأهمية للكشف عن هذه العدوى وإيجاد العلاج المناسب. وعليه تهدف هذه الدراسة إلى متابعة دقة وحساسية التقنيات التشخيصية المختلفة المستخدمة في الكشف عن داء البروسيلات في الحليب (التقنيات المناعية والبكتريولوجية والجزيئية). يعد اختبار حلقة الحليب (MRT)) ومقايسة الامتصاص المناعي المرتبط بالإنزيم غير المباشر (i-ELISA) ) من بين التقنيات المستخدمة حيث تم الكشف عن داء البروسيلات الإيجابي في عينات الحليب بنسب إجمالية قدرها ٣٤,٩١٪ و ٣٣,٩٦٪ على التوالي. بلغت الحساسية النسبية المسجلة لـ MRT و i-ELISA 93.55% و٩٦,٧٧٪ على التوالي، بينما كانت خصوصيتهما النسبية ٨٩,٣٣٪ و٩٢٪ على التوالي. تم العثور على i-ELISA لإظهار حساسية ونوعية نسبية أعلى من اختبار حلقة الحليب (MRT)). بلغت النسبة المئوية لأنواع البروسيلا المكتشفة في عينات الحليب عن طريق الاستزراع البكتريولوجي ٢٥,٥٪ وتم الإبلاغ عن أن جميعها هي B. melitensis biovar 3. من ناحية أخرى، تم اكتشاف ٢٨,٣٪ من العينات بشكل إيجابي في كل من عينات اختبار البلمره المتسلسل التقليدى واختبار البلمره المتسلسل ذو الوقت الحيقيقى (qPCR). وكانت الحساسيات النسبية ٨٧,١٠٪، ٩٦,٧٧٪، و٩٦,٧٧٪ للزراعة البكتريولوجية، وتقنيات PCR التقليدية، وqPCR، على التوالي. ذكرت جميع التقنيات الثلاثة خصوصيات نسبية بنسبة ١٠٠٪. إن أكثر الأنواع المكتشفة في حليب الأبقار والتي تعاني من اضطرابات تناسلية هي B. melitensis biovar 3. أظهرت كلتا تقنيتي تفاعل البوليميراز المتسلسل أعلى حساسية ونوعية نسبية بالإضافة إلى كونها تقنيات سريعة ومنخفضة المخاطر مما يجعل تفاعل البوليميراز المتسلسل مفضل تقنية لتشخيص داء البروسيلات.

**الكلمات الدالة**: الحليب، تقنيات التشخيص، الحساسية النسبية، الخصوصية النسبية.