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Comparison of real-time Polymerase Chain Reaction for Detection of *Brucella* in infected cows' milk with Milk Ring, indirect ELISA, and whey agglutination tests

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ABSTRACT

Brucellosis is one of the most important diseases affecting both humans and animals in most developing countries. This study aimed to evaluate the efficiency of different serological tests used for the diagnosis of Brucellosis on serum samples as well as immunoassays adapted to detect anti-*Brucella* antibodies in the milk whey. Besides, seeking the efficiency of the conventional bacteriological examination against real-time PCR for the direct detection of *Brucella* species in milk samples. A total of 105 cows that belonged to a private farm in the Giza governorate were selected. Out of these 105 cows, 65 cows were suffering from reproductive disorders, and the remaining 40 cows were in contact with animals. This farm had no history of brucellosis vaccination and was under brucellosis quarantine measurements. Out of the examined 105 serum samples by the serological tests, 28 (26.7%), 25 (23.8%), 20 (19%), and 23 (22%) cows' sera were reacted positively to the Buffered Acidified Plate Agglutination Test (BAPAT), Rose Bengal plate test (RBPT), Rivanol test (RIV.T) and Complement Fixation Test (CFT) respectively. On the corresponding picture, out of 105 whey (w) samples examined by wBAPAT, wRBPT, and wRiv.T, antibodies against *Brucella* were detected in 17 samples (16.2%), 14 samples (13.3%), and 11 samples (10.5%) by these tests respectively. The percent positive result of the examined milk samples was 20% by MRT (21 out of 105 milk samples), while milk-ELISA (m-ELISA) showed 18.1% positive results to Brucellosis (19 out of 105 milk samples). The real-time PCR identified *Brucella melitensis* in 12 milk samples out of the examined 105 cows' milk samples (11.4%). On the contrary, the conventional culture method identified nine (8.5%) *Brucella*. In conclusion, real-time PCR is sensitive, safe, and efficient for the direct detection of *Brucella* species in Milk samples. The serological tests identified a higher number of reactors than the corresponding whey serological tests.

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Introduction

Brucellosis is a severe, though neglected, zoonosis with widespread distribution and global impact **(Adone and Pasquali 2013)**. The disease consequences on public health, animal health, and the economy are considered not only in endemic developing countries but also, in the developing countries where the infection is re-emerging in wildlife **(Jansen *et al.* 2019)**

The disease is caused by a member of the Gram-negative bacteria that belongs to the genus *Brucella*. Brucellae are small, nonmotile, facultative anaerobic, intracellular, Gram-negative coccobacilli that show strong host preference **(Higgins *et al.* 2017).** Brucellosis is transmitted through direct and indirect contact with infected animals. Mostly, via ingestion and less frequently through the conjunctiva and inhalation routes. The World Health Organization (WHO) laboratory biosafety manual classifies *Brucella* in risk group III **(WHO 2004)**.

Brucellosis is readily transmissible to humans, causing acute febrile illness and undulant fever, which may progress to a more chronic form. It can also produce serious complications affecting the musculoskeletal, cardiovascular, and central nervous systems **(Corbel 2006)**. *Brucella* remains alive for varying periods after elimination from the animal body. This depends upon the environment and the surrounding conditions **(Dhanashekar *et al.* 2012).**

Milk is an important source of brucellosis in humans when they consume unpasteurized milk and under heat-treated milk and milk products. Several circumstances complicate the diagnosis of bovine brucellosis. All the serological tests which used in the diagnosis of brucellosis are not 100% reliable and have limitations under all epidemiological circumstances. Milk is considered an important specimen for the diagnosis of Brucellosis. Based on this fact, there are different panels of sensitive and specific screening tests that are used to detect antibodies against *Brucella* in milk **(Chand *et al.* 2005). S**uch as the Milk Ring Test (MRT), whey rose bengal Plate test (wRBPT), whey buffered acidified plate antigen test (wBAPAT), Whey Microtiter Agglutination test (wMAT), Whey Rivanol test, and milk indirect ELISA. Among those panels, the Milk Ring Test (MRT), is used as an efficient dairy herd screening test to determine the potential occurrence of brucellosis by testing cows' milk from the bulk tank **(Alton *et al.,* 1988)**. Animals from an MRT-positive herd should be individually retested by blood serological tests to identify infected ones **(Alton *et al.* 1988)**. The MRT depends on the presence of *Brucella* agglutinins in milk mainly IgA isotype **(Katz *et al.* 1976).**

Whey agglutination test has been considered an important test in detecting serologically negative animals under either chronic or recent infection where these negative animals may lead to difficult eradication of brucellosis **(Kaltungo *et al.* 2014)**.

Diagnosis of brucellosis is the keystone for its proper eradication and control. The diagnosis of brucellosis in dairy animals involves either the isolation of *Brucella* from milk samples or the detection of antibodies in serum or milk. However, these methods are not wholly satisfactory. Although bacteriological isolation is the gold standard for the diagnosis yet, it is a time-consuming procedure, and handling the micro-organism is hazardous. Serological methods are not conclusive because not all infected animals produce significant levels of antibodies and because cross-reaction with other bacteria can give false-positive results **(Alton *et al.* 1988)**. Molecular detection methods have been widely used for *Brucella* diagnosis in the last decades. Real-time PCR, which has less hazard and high sensitivity, has been developed for *Brucella* detection. Real-time PCR does not require extensive manipulation that minimizes the risk of contamination **(Yaran *et al.* 2016).**

This study aimed to evaluate the efficiency of different serological tests used for the diagnosis of Brucellosis on serum samples (BAPAT, RBPT, Riv.T, and CFT) as well as immunoassays adapted to detect anti-*Brucella* antibodies in the milk and whey (wBAPAT, wRBPT, wRiv.T, m-ELISA, and MRT). Besides, seeking the efficiency of the

conventional bacteriological examination against Real-time PCR for the direct detection of *Brucella* species in milk samples.

Materials and Methods
1- Animals and Sampling: -
In the present study, a total of 105 cows that belonged to a private farm in the Giza governorate were selected. Of these 105 cows, some were suffering from reproductive disorders (n=65), and the remaining were in contact animals (n=40). This farm had no history of brucellosis vaccination and was under brucellosis quarantine measurements.

Collection of blood samples:
The blood samples were collected from the Jugular vein after disinfecting the site of injection by ethyl alcohol 70%. About 8-10 ml of blood is collected from each animal in a plain vacutainer tube. The samples were coded and transferred to the laboratory with minimal delay in an insulated icebox.

Collection of milk samples:
During the collection of milk samples, the whole udder and teats of the cows were washed and dried. About 10ml of milk was collected in sterile screw-capped McCartney bottles after discarding the first stream of milk from all quarters. The collected milk samples were subdivided into three parts. one for bacteriological isolation, the second for PCR assay, and the third for Milk ring test (MRT), ELISA, and preparation of whey for whey-based immunoassays (wBAPAT, wRBPT, and wRiv. T).

2- Examination of collected sera samples: -
For serological examination of serum, sera were prepared according to (**Alton** *et al.* **1988 OIE 2019**).
1. Buffered acidified plate antigen test (BAPAT): The test was performed according to (**Anon 1984 OIE 2019**), where any visible agglutination within 8 minutes is considered positive. No agglutination within 8 minutes was regarded as negative.
2. Rose-Bengal plate test (RBPT): The test was conducted according to (**Alton** *et al.* **1988 OIE 2019**), where any visible agglutination

within 4 minutes was considered positive. No agglutination within 4 minutes was regarded as negative.
3. Rivanol test: The test was carried out according to (**Alton** *et al.* **1988 OIE 2019**). Any sample exhibiting complete agglutination at 1:25 or higher reaction was considered as positive.
4. Complement Fixation test (CFT): The test was performed according to (**Alton** *et al.* **1988; OIE 2019**) using G. Pig serum as a source of complement and a pretreated amount of 3% sensitized sheep erythrocytes. Sera were considered positive when it gives a positive fixation at a titer of 1/4 or higher ($\geq$20 IU/mL).

3-Examination of collected milk samples:
Milk whey samples were prepared according to (**Morgan** *et al.* **1978**). Milk whey was prepared by the addition of 200 µl of commercial liquid rennet (strength 1: 10000; 0.3%, w/v) to 10 ml of each milk sample and incubation at 37°C for 30 min until the coagulation occurred and followed by removal of the casein by filtration and centrifugation. Clear milk whey was collected and stored at -20°C until use.

The whey was treated as serum in the following tests:
1. Whey Buffered Acidified Plate Antigen Test (wBAPAT) (*Alton et al.* **1988**).
2. Whey Rose Bengal Plate Test (wRBPT) (**Alton** *et al.* **1988**).
3. Milk Ring Test (**Alton** *et al.* **1988; OIE 2019**).
MRT: The procedure described by **Alton** *et al.* **(1988) and OIE (2019)** was used in the MRT. MRT antigen; a hematoxylin stained 4% packed *B. abortus* strain 99 cells in citrate phosphate buffer (pH 3.3-3.7) was supplied by Animal Health Veterinary Laboratories Agency (AHVLA), DEFRA, UK. The test was considered negative if the color of the underlying milk exceeds that of the cream layer.
Positive results are given scores as follows: Dark blue cream ring with white milk column was given (++++), definitely blue cream ring with slightly blue milk column was given (+++), definitely blue cream ring with moderately blue milk column was given (++), cream ring with milk column about the

same color was given (+).

4. Indirect Enzyme-Linked Immunosorbent Assay (m-ELISA) on milk: The indirect ELISA (m-ELISA) kit for detection of anti-*Brucella* antibodies was obtained from M/S Svanova, Sweden, and the procedure was followed as per the manufacturer's procedure. Milk samples were centrifuged at 5000 rpm for 5 minutes to remove the cream layer before being tested by the iELISA kit. The interpretation of the results was performed accordingly. Percent positivity values (PP) = Test sample OD ×100/ mean positive control OD. For milk samples as ascribed by the manufactural company, a percent positivity (PP) value equal to or more than 10 was taken as positive for m-ELISA.

4-Bacteriological Examination of milk Samples:

Briefly, the milk sample was centrifuged at 3000 rpm for 10 minutes to obtain the sediment–cream mixture (**Alton *et al*. 1988; OIE 2019**) which, then was cultured on duplicated plates of *Brucella* selective medium supplemented with antibiotics (Oxoid, UK). The plates were incubated in presence of 5-10% CO2 and normal air condition at 37 °C for up to 2 weeks. The *Brucella* isolates were fully typed at the genus, species, and biovar levels through the method adopted by **Alton *et al*. (1988).**

5-Molecular diagnosis:

DNA extraction. DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

B. Oligonucleotide Primers. Primers used were supplied from bio basic (Canada) and are listed in the (**Table 1**).

Table (1): Primers sequences, target gene, and cycling conditions for SYBR green rt-PCR.

Target gene	Target agent	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (40 cycles)			Dissociation curve (1 cycle)		
					Secondary denaturation	Annealing (Optics on)	Extension	Secondary denaturation	Annealing	Final denaturation
1S711	*B. melitensis*	1S711-specificPrimer TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT *B. melitensis*-specific Primer AAA-TCG-CGT-CCT-TGC-TGG-TCT-GA	731	94°C 15 min.	94°C 15 sec.	55°C 30 sec.	72°C 45 sec.	94°C 1 min.	55°C 1 min.	94°C 1 min.

C. SYBR green rt-PCR. Primers were utilized in a 25- µl reaction containing 12.5 µl of the 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 1 µl of each primer of 20 pmol concentration, 5.5 µl of water, and 5 µl of DNA template. The reaction was performed in a Stratagene MX3005P real-time PCR machine.

D. Analysis of the SYBR green rt-PCR results. Amplification curves and ct values were determined by the Stratagene MX3005P software.

Brucella IS711 species specific real-time PCRs was performed for using *B. melitensis* primers listed in the table (1) after **Bricker and Halling (1994).**

Optimization obtained through the dissociation curve achieved by Secondary denaturation (1 minute at 94˚C), Annealing (1 minute at 55˚C), and Final denaturation (1 minute at 94˚C) as shown by **Table 1**. The samples considered positive if the DNA amplification exceeded the threshold line limit.

RESULTS

As shown by **Table (2)**, our study revealed the presence of the antibodies against *Brucella* organisms in 28 (26.7%), 25 (23.8%), 20 (19%), and 23 (22%) cows' sera by Buffered Acidified Plate Agglutination Test (BAPAT), Rose Bengal plate test (RBPT), Rivanol test (RIV.T) and Complement Fixation Test (CFT) respectively.

The overall percent positive of brucellosis in examined animals was 20% by MRT, as 21 out of 105 samples were found positive while milk-ELISA (m-ELISA) showed 18% seropositive results, as 19 out of 105 milk samples were found positive by the test **(Table 3).** Out of 105 whey (w) samples examined by wBAPAT, wRBPT, and wRiv.T, antibodies against *Brucella* were detected in 17 samples (16.2%), 14 samples (13.3%), and 11 samples (10.5%) by these tests respectively.

Table (2) and Table (3) revealed that every serological test, under the field of this study, gave higher positive reactors than its corresponding serological test done on milk whey.
The PCR assay detected and identified more *Brucella* positive samples than those by the

culture method **(Table 4)**. It detects *Brucella* DNA in eight out of 65 (9.2%) in the group where animals suffered from reproductive disorders and even in the apparently healthy group (4 out of 40;10%). On the contrary, culture is positive in lower numbers of examined animals than PCR in the same comparing groups (6 out of 65 (9.2%) in the group of reproductive disorder suffering animals; 3 (7.5%) out of 40 apparent healthy cows.
SYBR green RT-PCR targeting the IS711 gene amplified the *Brucella melitensis* genomic DNA in 11.4% of milk samples (12 out of the examined 105 cows' milk samples) as shown by **Figure (1)**.

DISCUSSION

Abortion is a frequent complication of brucellosis in animals. Placental localization by *Brucella* is believed to be associated with the *Brucella* growth stimulant erythritol **(Corbel 2006).** It has been postulated that generalized suppression of adaptive immune response mainly occurred during pregnancy. This immune-suppressed state prevents maternal rejection of the fetus but has the unfortunate consequence of increasing maternal susceptibility to certain infectious agents **(Krishnan *et al*. 1996).**

The BAPAT test detected a higher number of positive as compared with other serological tests **(Table 2)**, and this attributed to the final packed cell volume (3%) and the final pH (3.8) of a serum antigen mixture **(Alton *et al*. 1988),** this acidic pH reduces the agglutination ability of IgM besides, enhancing the agglutination of IgG1 **(Wright and Nielsen 1990)**. Furtherly **OIE (2019)** reported that buffered *Brucella* antigen tests are suitable for screening herds and individual animals.
The RBPT detected immunoglobulin against *Brucella* in 25 (23.8%) out of the examined 105 cows' sera, slightly lower than the BAPAT positive samples **(Table 2)**. The RBPT detects antibodies of classes IgG1 and IgM which, are provoked against surface antigen lipopolysaccharides (LPS) of smooth *Brucella* **(Davies 1971).** RBPT is recommended by the OIE for international trade and is a test of choice for the screening of ruminant brucellosis **(Garin-Bastuji and Blasco 2004).**

Serological examination performed by RB test in the present study gave a positive result in some aborted animals but were negative for Riv.T **(Table 2).** This diversity may attribute to the cross-reactions with related heterospecific antigens of Gram-negative bacteria, e.g. *Yersinia enterocolitica* O:9, *E. coli* O:157, and *Pseudomonas maltophilia* resulted in false-positive reactions **(Nicoletti 1984).**

The Rivanol test detected positive reactors lower than those detected by BAPAT or RBT as shown in (**Table 2**). Our result is in agreement with those reported by **Hosein *et al.* (2002)** and **Abdel-Hamid *et al.* (2017)**.

The lower number of Brucellosis reactors recorded by the Rivanol test than both BAPAT and RBT may be attributed to the precipitation of IgM, responsible for the non-specific reaction, during the first step of the test (treating the serum samples with the Rivanol solution; 2 – ethoxy- 6, 9 diamino acridine lactate). This rivanol solution, cationic acridine dye, forms a complex with high molecular weight serum glycoproteins (serum IgM). IgM carries a relatively high net negative charge compared to IgG which, has low or negligible net negative charges at neutral pH. This complex easily precipitates by centrifugation. Precipitation of IgM class of antibodies eliminates the non-specific agglutination from bovine serum as reported by **Pietz and Goward (1980)**.

In this research, examination of serum samples by CFT led to seropositivity being detected in 23 (22%) samples out of the examined serum samples (n=105).

CFT is considered the most specific serological test as it detects only IgG1 specific for *Brucella* long-lasting infection. CFT is recommended by the OIE to contribute to the eradication policies and to incorporate herd/flock surveillance of brucellosis. Additionally, the World Organization for Animal Health (OIE) considered CFT a suitable method to confirm *Brucella* suspected or clinical cases **(OIE 2019)**. This test is considered a high-quality test when correctly used, however, it has many practical drawbacks such as being time-consuming and difficult to standardize **(Abernethy *et al.* 2012).**

The consumption of contaminated milk and milk products is one of the main transmission ways of pathogenic *Brucella* strains to humans. For that reason, a fast and accurate evaluation of the brucellosis status of the milk and its products is paramount relevant for public health.

In the present study, the MRT had detected more positive cases than milk ELISA (**Table 3**). These findings of corroborate with **Kerkhofs *et al.* (1990)** who reported nearly similar results. However, **Nivedi (2014),** reported a high prevalence of MRT (7.38%) than m-ELISA **(6.04%)**. **Vanzini *et al.* (2001), Shafee *et al.* (2011),** and **Mohamed (2015)** suggested that the m-ELISA was more sensitive than MRT
MRT is the most widely used test for monitoring and screening brucellosis in dairy cattle **(Alton *et al.* 1988).** A high positive result with MRT could be due to various milk conditions such as mastitis, colostrum, in milk samples collected shortly after parturition, near the end of lactation, mastitis cows, or vaccinated animals **(OIE 2019).**

Serological tests such as MRT and ELISA are widely used for the detection of anti-*Brucella* antibodies in milk. However, MRT often causes false-positive results and its sensitivity and specificity have been found lower than ELISA methods **(Nielsen 2002)**. **(Hermoon *et al.* 2001)** reported that ELISA is a suitable test for large-scale screening for Bovine brucellosis. Besides latent infection could be detected earlier by ELISA than other serological tests as it detects all classes of antibodies.

Our study revealed that every serological test gave higher positive reactors than its corresponding serological test done on milk whey (**Table 2 and3**). This could be attributed to the defatting process done during whey preparation since most of the immunoglobulins are present on the surface of the fat globules

(**Hamdy 1997**). Removal of solid parts by rennin during whey preparation, changes in the pH, and the molecular weight of immunoglobulins could be, additional factors of the low sensitivity of the whey agglutination tests (**Hamdy 1997 Abdel-Hamid *et al.* 2008**).

The prevalence of *Brucella* contamination varies according to the sensitivity of the used methods. Although isolation and phenotyping of *Brucella* are time-consuming and unsafe and need well-trained staff, they are still the gold standard for diagnosis of *Brucella* spp. Today by using molecular detection techniques like PCR, the detection of brucellosis is significantly increasing (**Yu and Nielsen 2010**). Indeed, several studies have shown that agents such as lipids, enzymes, polysaccharides, proteins, and Ca2+ in high concentration that are present in dairy products can play the role of PCR inhibitor by interfering with nucleic acid degradation or with the amplification activity of polymerase (**Lindahl-Rajala *et al.* 2017**).

Unlike the circulating bacteria and DNA, antibodies against *Brucella* antigen remain in the blood for a long time, making sometimes PCR results negative while ELISA results are positive and that's matching our results (**Table 3 and 4**).

The higher sensitivity of PCR assay than the culture method in this study (**Table 4**) may be attributed to the high sensitivity of PCR to detect the fewer number of *Brucella* organisms present in the milk sample than that can be detected by culture (**Leal-Klevezas *et al.* 1995 Hamdy and Aminy 2002**). Also, PCR detects DNA which present in both living and dead *Brucella* organisms while culture detects only the living organisms (**Hamdy and Aminy 2002**) and/or to occurrence of false-negative bacteriological results due to massive contamination of the milk samples or from inhibition of some *Brucella spp.* in the selective medium that is the major factor that limits the use of conventional bacteriological methods. In these circumstances, *Brucella* DNA can still be detected by PCR assay (**Romero *et al.* 1995**). Here, it must be mentioned that the survival of *Brucella* organisms in milk is altered by the change in pH and storage temperature (**Hamdy 1992**) and that *Brucella* content of milk depends on the stage of the lactation (**El- Berg, 1981**) and infection (**Romero *et al.* 1995**). **Romero et al. (1995) and O'Leary *et al.* (2006)** attributed the lower sensitivity of PCR assay than culture to the presence of several *Brucella* organisms below the detection limit of the used PCR, degradation of target DNA in the sample (s), and/or to the presence of polymerase inhibitors in the milk sample (s) and using inefficient *Brucella* DNA extraction protocol. The possibility of PCR techniques to detect the DNA of dead bacteria, even in highly contaminated samples with other microorganisms, could potentially increase the rate of pick-up animals infected with *Brucella* (**Garin-Bastuji *et al.* 2006**)

The sensitivity of the *Brucella* culturing depends on 1-the viability and numbers of *Brucella* within the sample. 2-the nature of the sample. 3-the number of specimens tested from the same animal. The time required for culturing field specimens is long, and tissues or fluids that are only contaminated with a low number of *Brucella* may not be detected. Thus, in the case of tissues or fluids contaminated with non-viable or a low number of *Brucella*, real-time PCR could be a potentially useful method for the diagnosis of brucellosis (**Wareth *et al.* 2014).**

The assessment of real-time PCR technique for detection of *B. melitensis* in non-pasteurized milk was done by **Wareth *et al.* (2014)**. They notified that non-pasteurized dairy product are important sources for the prevalence of brucellosis and real-time PCR is qualified and efficient for detection of this pathogen. As mentioned earlier, one of the most important ways for brucellosis infection transmission is the consumption of infected dairy products. The results of this study showed that 11.4% of milk is infected with *Brucella* spp by using real-time PCR.

Despite this fact, the consumption of non-pasteurized dairy products in many places still makes a great concern for the disease prevalence. Therefore, reducing the possibility of being infected by this pathogen by using accurate molecular detection techniques like real-

time PCR should be considered (**Garshasbi *et al.* 2014**).

By comparing results of PCR assay with culture method for isolation and identification of B*rucella* organisms recovered from cows' milk samples as shown in (Table 4 and Figure 1) the study revealed that PCR assay detected and identified more *Brucella* positive samples than those by culture method. The real-time PCR detected and identified *B. melitensis* in 12 (11.4%) milk samples out of the examined 105 cow's milk samples, while the convention culture method identified only 9(8.5%) out of the examined 105 cow's milk samples **(Table 4 and Figure 1)**. All the *Brucella* isolates were identified as *B. melitensis* bv3. These findings agree with **Foster *et al.* (2017)** who reported that the limitations of both isolation and serological detection procedures have resulted in increasing the use of PCR-based methods for detection and identification of *Brucella* species due to their accuracy, sensitivity, speed, and ability to work with DNA as opposed to highly infectious live cultures Unlike finding of this study, **Romero *et al.* (1995)** and **O'Leary *et al.* (2006)** found that culture method detected more positive samples than PCR assay.

Molecular diagnostics have the edge over conventional methods as they are robust and versatile, and due to the non-infectious nature of DNA, therefore safer for laboratory personnel. Despite the availability of newly developed techniques, the isolation and identification of the agents by cultural techniques are still the gold standard for the diagnosis of brucellosis (**Unver *et al.* 2006).**

Overall, the isolates from animals of Middle Eastern countries were dominantly determined as *B. melitensis* biovar 3, which is the result parallel to those obtained in the present study (**Refai 2002 Abdel-Hamid *et al.* 2021**). The diagnosis of brucellosis is challenging as culturing of Brucellae and seroconversion are time-consuming. Therefore, molecular techniques like real-time PCR are promising alter-

natives for the diagnosis of infectious diseases caused by fastidious or slowly growing micro-organisms such as Brucellae.

In conclusion

The real-time PCR is qualified, safe, and efficient for the direct detection of *Brucella* species in Milk samples. The serological tests identified a higher number of reactors than the corresponding whey serological tests. The milk -ELISA and MRT, for Brucellosis, appear to be technically sound as dairy herd screening tests.

Table (2): Results of different serological tests for cow's serum.

Animal status	No. of sample	BAPAT		RBPT		Riv. T		CFT	
		No.	%	No.	%	No.	%	No.	%
reproductive disorders	65	18	**27.7**	16	**24.6**	13	**20**	15	**23.1**
apparently healthy	40	10	**25**	9	**22.5**	7	**17.5**	8	**20**
Total	105	28	**26.7**	25	**23.8**	20	**19**	23	**22**

BAPAT = Buffered Acidified Plate Antigen Test. RBPT= Rose Bengal Plate Test.
TAT= Tube Agglutination Test. Riv. T= RivanolT

Table (3): Results of Milk ELISA, milk ring test and different serological tests applied on cow's milk samples

Animal status	No. of sample	W BAPAT		W RBPT		W Riv.T		MRT		m-ELISA	
		No.	%	No.	%	No.	%	No.	%	No.	%
reproductive disorders	65	11	**17**	10	**15.4**	8	**12.3**	14	**21.5**	13	**20**
apparently healthy	40	6	**15**	4	**10**	3	**7.5**	7	**17.5**	6	**15**
Total	105	17	16.2	14	13.3	11	10.5	21	20	19	18.1

wBAPAT=whey Buffered Acidified Plate Antigen Test. wRBPT= whey Rose Bengal Plate Test. wRiv. T = whey Rivanol Test. MRT = Milk Ring Test.

Table (4): Detection of *Brucella* organisms in milk samples by culture method and real-time PCR assay

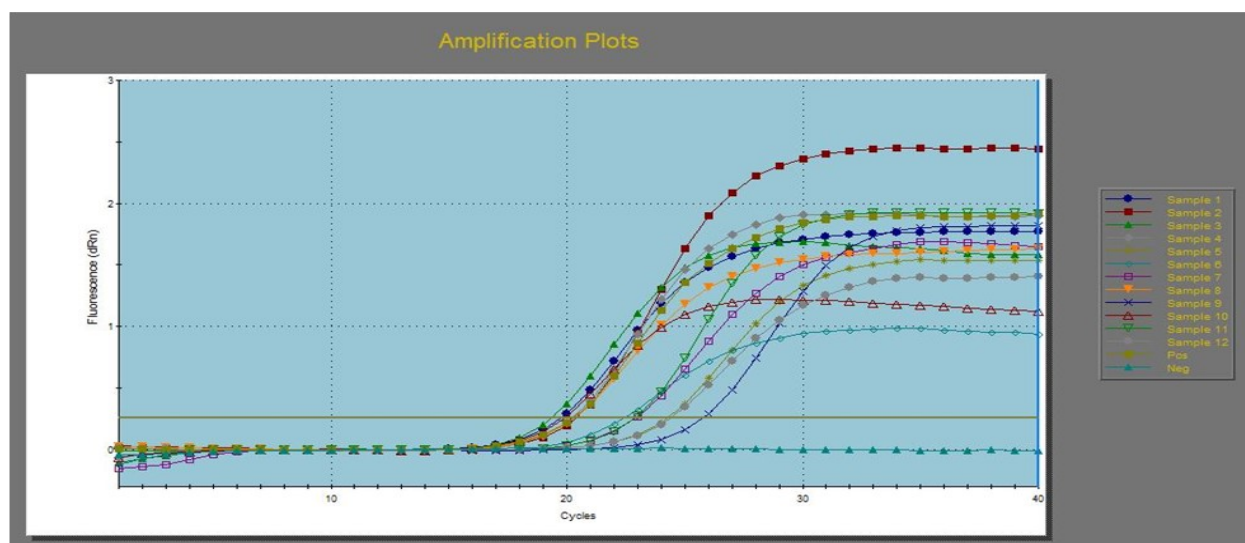| Animal status	No. of samples	Positive samples			
		Culture		Real- time PCR assay	
		No.	%	No.	%
reproductive disorders	65	6	9.2	8	12.3
apparently healthy	40	3	7.5	4	10
Total	105	9	8.5	12	11.4

Figure (1) Amplification curves of SYBR green real-time polymerase chain reaction for the detection of the DNA of the *Brucella melitensis* recovered from infected lactating cows.

REFERENCES

Abdel-Hamid NH, El-bauomy EM, Ghobashy HM, Shehata AA. 2021. Genetic variation of *Brucella* isolates at strain level in Egypt. Vet Med Sci. (6): 421–432. https://doi. org/10.1002/vms 3.260.

Abdel-Hamid NH, Shell WS, Khafagi MHM. 2017. Comprehensive Serological, Bacteriological, and Molecular Perspective of Brucellosis in Cattle and Buffaloes in Some Governorates. International Journal of Recent Scientific Research, 8(9): 20226–20234. https://doi.org/10.24327/ijrsr.2017. 0808. 0853.

Abdel-Hamid RF, Mohammed IS, Ahmed SA, Hassan EA, El-Bauomy EEM. 2008. Studies on *Brucella* in milk and some milk products in Assiut city. M.V.Sc. Thesis, Fac. of Vet. Med., Assiut Uni., Egypt.

Abernethy DA, Menzies FD, McCullough SJ, McDowell SW, Burns, KE, Watt R, Gordon AW, Greiner M, Pfeiffer DU. 2012. Field trial of six serological tests for bovine brucellosis. Vet. J. 191(3):364-370.https://doi.org/10.1016/j.tvjl.2011.03.008.

Adone R, Pasquali P. 2013. Epidemio-surveillance of brucellosis. Rev. sci. tech. 32 (1):199–205. https://doi.org/10.20506/rst. 32. 1. 2202.

Alton GG, Jones LM, Angus RD, Verger JM. 1988. Techniques for the Brucellosis Laboratory. Institut National de la Recherche Agronomique Publications, Paris, France.

Anon L. 1984. Instruction for conducting brucellosis serological tests, National Veterinary service Laboratories, Ames Iowa, USA

Bricker BJ, Halling SM. 1994. Differentiation of *Brucella abortus*bv. 1, 2 and 4, *Brucella melitensis*, *Brucella ovis* and *Brucella suis*bv. 1 by PCR. J. Clin. Microbiol. (32): 2660-2666.

Chand P, Rajpurohit BS, Alhotra, MAK, Poonia JS. 2005. Comparison of milk-ELISA and serum-ELISA for the diagnosis of *Brucella melitensis* infection in sheep. Vet. Microbiol. (108): 305-311. https://doi.org/10.1016/j.vetmic. 2005.04.006.

Corbel MJ. 2006. Brucellosis in humans and animals. The World Health Organization, in collaboration with the Food and Agriculture Organization of the United Nations and the World Organization for Animal Health (p. WHO/CDS/EPR/2006.7.). Geneva: WHO Press.

Davis G. 1971. The Rose Bengal Test. Vet. rec. (88): 447-449.

Dhanashekar R, Akkinepalli S, Nellutla A. 2012. Milk-borne infections. An analysis of their potential effect on the milk industry. Germs.2(3):101–109.https://doi. org/10.11599/germs.2012.1020.

El-Berg SS. 1981. A guide to diagnosis, treatment and prevention of human brucellosis. WHO, VPH/81-31.

Foster JT, Walker MF, Rannals DB, Hussain HM, Drees PK, Tiller VR, Hoffmaster RA, Al-Rawahi A, Keim P, Saqib M. 2017. African lineage *Brucella melitensis* isolates from Omani livestock. Front. Microbiol. 8: 2702.https://doi.org/10.3389/ fmicb.2017.02702.

Garin-Bastuji B, Blasco J, Marin C, Albert D. 2006. The diagnosis of brucellosis in sheep and goats, old and new tools. Small Ruminant Res, (62): 63-70. https://doi.org/10.1016/j.smallrumres. 2005.08.004.

Garin-Bastuji B, Blasco JM. 2004. Caprine and ovine brucellosis (excluding B. ovis). In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, fifth ed. OIE, pp. 598–606.

Garshasbi M, Ramazani A, Sorouri R, Javani S, Moradi S. 2014. Molecular detection of *Brucella* species in patients suspicious of Brucellosis from Zanjan, Iran. Braz J Microbiol. 45(2):533-8. https://doi.org/10.1590/ s1517-83822014005000048.

Hamdy ME. 1997. Correlation between humoral and cellular immune response in dairy cows naturally infected with *Brucella abortus*, J. Egypt Vet. Med. Assoc. 57(1): 331-349

Hamdy ME, Aminy AS. 2002. Detection of *Brucella* species in the milk of infected cattle, sheep, goats and camels by PCR. Vet. J., (163): 299-305. https://doi.org/10.1053/tvjl. 2001.0681.

Hamdy MER. 1992. Epidemiological studies on *Brucella melitensis* in dairy animals and man. Ph. D. Thesis, Faculty of Veterinary Medicine, Cairo University, Cairo.

Hermoon CD, Jung B, Cho S, Jung S, Kim O. 2001. Development of ELISA for *Brucella*

abortus RB51. Purification of 8 KDa antigen and development of ELISA using its antigen of *Brucella abortus*. Korean J. Vet. Res. (41): 51-57.

Higgins JL, Gonzalez-Juarrero M, Bowen RA. 2017. Evaluation of shedding, tissue burdens, and humoral immune response in goats after experimental challenge with the virulent *Brucella melitensis* strain 16M and the reduced virulence vaccine strain Rev. 1. PLoS One. 12: e0185823, https://doi.org/10.1371/journal.pone.0185823.

Hosein HI, Dawood FZ, El-Sheery MN. 2002. Evaluation of the policy of test and slaughter for control of brucellosis in Egypt. 10th Sci. Con., Fac. Vet. Med., Assiut University, Egypt.

Jansen W, Linard C, Noll M, Nöckler K, Al Dahouk S. 2019. *Brucella*-positive raw milk cheese sold on the inner European market: A public health threat due to illegal import? Food Control. 100: 130–137, https://doi.org/10.1016/j.foodcont..01.022.

Kaltungo BY, Saidu SNA, Sackey AKB, Kazeem HM. 2014. A review on diagnostic techniques for brucellosis. Afr. J. Biotechnol. 13 (1): 1-10. https://doi.org/10.5897/AJB2013.13442.

Katz KW, Greathhead MM, Cook RC. 1976. Experience in the diagnosis of brucellosis in dairy cows. J. S. Afr. Vet. Assoc. (47): 97-100.

Kerkhofs P, Botton Y, Thiange P, Dekeyser P, Limet JN. 1990. Diagnosis of bovine brucellosis by enzyme immunoassay of milk. Vet Microbiol. 24(1):73-80. https://doi.org/10.1016/0378-1135(90)90052-w.

Krishnan L, Guilbert L, Russell AS, Wegmann TG, Belosevic M. 1996. Pregnancy impaire resistance C57Bl/6 mice to Lishmania major infection and cause decrease antigenic specific IFN-gamma response and increased production of T helper 2 cytokines. J. Immunol. (1156): 644-651.

Leal-Klevezas DS, Martínez-Vázquez IO, López-Merino A, Martínez-Soriano JP. 1995. Single-step PCR for detection of *Brucella* spp. from blood and milk of infected animals. J Clin Microbiol. 33(12):3087-90. https://doi.org/10.1128/jcm.33.12.3087-3090.1995.

Lindahl-Rajala E, Hoffman T, Fretin D, God-

froid J, Sattorov N, Boqvist S, Lundkvist, A, Magnusson U. 2017. Detection and characterization of *Brucella* spp. in bovine milk in small-scale urban and peri-urban farming in Tajikistan. PLoSNegl Trop Dis. 11(3): e0005367.https://doi.org/10.1371/journal.pntd.0005367.

Mohamed NA. 2015. The prevalence of Bovine Brucellosis Using Four Serological Tests in Khartoum State. M.Sc., thesis submitted to Graduate College, Khartoum.

Morgan WJB, Mackinnon DJ, Gill KPW, Cower SGM, Norris PLW. 1978. Standard laboratory techniques for the diagnosis of brucellosis. Repot Series No., I, Weybridge Cent. Vet. Lab., England.

Nicoletti P. 1984. Diagnosis and Vaccination for the control of brucellosis in the Near East. FAO, Ani. Prod. And Hlth paper. Series No. 38, Rome.

Nielsen K. 2002. Diagnosis of brucellosis by serology. Vet Microbiol. (90):447–59. https://doi.org/10.1016/S0378-1135(02)00229-8.

Nivedi 2014. National Institute of Veterinary Epidemiology and Disease Informatics, Annual report, 2013-14, Hebaal, Bangalore, pp. 42.

O'Leary S, Sheahan M, Sweeney T. 2006. *Brucella abortus* detection by PCR assay in blood, milk and lymph tissue of serologically positive cows. Res. Vet. Sci. (81):170–176.https://doi.org/10.1016/j.rvsc.2005.12.001.

OIE, Terrestrial Manual. Ch. 3.1.4. Paris: OIE; 2019. Infection with *Brucella abortus*, *Brucella melitensis* and *Brucella suis*; pp. 355–398.

Pietz DE, Cowart WD. 1980. Use of epidemiological data and serologic test in bovine brucellosis. J. Am. Vet. Med. Ass. (77):1221-1226

Refai, M. 2002. Incidence and control of brucellosis in the Near East Region. Vet. Microbiol., (90): 81–110. https://doi. org/10. 1016/s0378-1135(02)00248-1.

Romero C, Pardo M, Grillo MJ, Diaz R, Blasco JM, Lopez-Goni I, Goni IL. 1995. Evaluation of PCR And Indirect Enzyme linked Immunosorbent Assay on Milk Samples for Diagnosis of Brucellosis in Dairy Cattle. Journal Of Clinical Microbiology 33, 3198

Shafee M, Rabbani M, Sheikh AA, Ahmad MD, Razzaq A. 2011. Prevalence of Bovine Brucellosis in Organized Dairy Farms, Using Milk ELISA, in Quetta City, Balochistan, Pakistan. Vet. Med. Int., Article ID 358950, 3 pages https://doi.org/10.4061/2011/358950.

Unver A, Erdoğan H, Atabay H, Şahin M, Çelebi Ö. 2006. Isolation, identification, and molecular characterization of *Brucella melitensis* from aborted sheep fetuses in Kars, Turkey. Rev. Med. Vet. 157(1): 42-46.

Vanzini VR, Aguirre NP, Valentini BS, Torioni de Echaide S, Lugaresi CI, Marchesino MD, Nielsen K. 2001. Comparison of an indirect ELISA with the *Brucella* milk ring test for detection of antibodies to *Brucella abortus* in bulk milk samples. Vet Microbiol. 82 (1):55-60.   https://doi.org/10.1016/s0378-1135(01)00370-4.

Wareth G, Melzer F, Elschner MC, Neubauer H, Roesler U. 2014. Detection of *Brucella melitensis* in bovine milk and milk products from apparently healthy animals in Egypt by real-time PCR. J Infect Dev Ctries. 8 (10):1339-43.https://doi.org/10.3855/jidc. 4847.

WHO 2004 Laboratory biosafety manual, 3rd ed. World Health Organization. https:// apps.who.int/iris/handle/10665/42981

Wright PF. and Nielsen KH. 1990. Current and future serological methods. In: Garry Adams, L. (editor): Advances in brucellosis Research. Texas A&M University Press, Texas, USA, pp. 305- 320.

Yaran M, Najafi S, Shoaei P, Ataei B, Fadaei R, Ramazanpour J, Mehdi F, Nasrin AA, Marzieh K. 2016. Prevalence of *Brucella melitensis* and *Brucella abortus* in raw milk and dairy product by real time PCR technique. Ulutas Med J. 2(1):7-11. https:// doi.org/10.5455/umj.20151004054235.

Yu WL, Nielsen K. 2010. Review of detection of *Brucella* spp. by polymerase chain reaction. Croat Med J. 51(4):306-13. https:// doi.org/10.3325/cmj. 2010.51.306.