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.
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). Such 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 1999), 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 (∆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.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Target agent</th>
<th>Primers sequences</th>
<th>Amplified segment (bp)</th>
<th>Primary denaturation</th>
<th>Secondary denaturation</th>
<th>Annealing (Optics)</th>
<th>Extension</th>
<th>Secondary denaturation</th>
<th>Annealing</th>
<th>Final denaturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1S711</td>
<td><em>B. melitensis</em></td>
<td>1S711-specific Primer TGC-CGA-TCA- CTT-AAG-GGC- CTT-CAT</td>
<td>731</td>
<td>94°C 15 min.</td>
<td>94°C 15 sec.</td>
<td>55°C 30 sec.</td>
<td>72°C 45 sec.</td>
<td>94°C 1 min.</td>
<td>55°C 1 min.</td>
<td>94°C 1 min.</td>
</tr>
</tbody>
</table>

\[B. melitensis\text{-specific Primer AAA-TGC-CGT- CCT-TGC-TGG- TCT-GA}\]
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 milk samples were found positive by the test (Table 3). Out of 105 milk samples were found positive by the test (Table 3). Out of 105 milk samples were found positive by the test (Table 3). Out of 105 milk samples were found positive by the test (Table 3). Out of 105 milk samples were found positive by the test (Table 3). Out of 105 milk samples were found positive by the test (Table 3). Out of 105 milk samples were found positive by the test (Table 3). Out of 105 milk samples were found positive by the test (Table 3). Out of 105 milk samples were found positive by the test (Table 3). Out of 105 milk samples were found positive by the test (Table 3). Out of 105 milk samples were found positive by the test (Table 3). Out of 105 milk samples were found positive by the test (Table 3). Out of 105 milk samples were found positive by the test (Table 3). Out of 105 milk samples were found positive by the test (Table 3). Out of 105 milk samples were found positive by the test (Table 3).

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

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

BAPAT = Buffered Acidified Plate Antigen Test. RBPT = Rose Bengal Plate Test. Riv. T = Rivanol T

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

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

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.

<table>
<thead>
<tr>
<th>Animal status</th>
<th>No. of samples</th>
<th>Positive samples</th>
<th>Culture No.</th>
<th>%</th>
<th>Real- time PCR assay No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>reproductive disorders</td>
<td>65</td>
<td>6</td>
<td>9.2</td>
<td>8</td>
<td>12.3</td>
<td></td>
</tr>
<tr>
<td>apparently healthy</td>
<td>40</td>
<td>3</td>
<td>7.5</td>
<td>4</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>105</td>
<td>9</td>
<td>8.5</td>
<td>12</td>
<td>11.4</td>
<td></td>
</tr>
</tbody>
</table>

**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.
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