Detection of Brucella organisms from Egyptian raw milk using cultural and molecular techniques

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Abstract

Aim: This study aimed to detect Brucella organisms in Egyptian raw milk using cultural and molecular techniques.

Methods: Two hundred bulk milk samples were collected from 2 cities at El-Gharbia and Kafer El-Sheikh Governorates during the summer and winter seasons. All samples were examined by the traditional cultural method and real-time polymerase chain reaction (qRT-PCR). All positive isolates were identified by biochemical tests and serotyping using monospecific antisera also examined by multiplex PCR primers for more identification.

Results: Samples collected in summer seasons from the two cities showed no colonies characteristic to Brucella organisms while in winter season five samples showed positive characteristic features of Brucella organisms after culturing on Brucella specific media. Using qRT-PCR, 43 samples (13 in summer and 30 in winter) were positive for Brucella organisms. All positive RT-PCR samples and isolated strains were examined for the detection of the virulence genes Ure, bvfA and virB using conventional PCR. Ure, bvfA and virB were detected in 88%, 79% and 74% of milk samples and 100%, 80% 100% of isolates, respectively.

Conclusion: These results suggested that the use of the specific PCR assay is a rapid, reliable, and accurate technique in comparison to traditional methods for the detection of Brucella species in milk samples.

Keywords: Milk, Brucella, Culture, PCR, Virulence genes.

1. Introduction

Raw milk is a natural biological fluid obtained from healthy udders of lactating cows. It provides compounds that play an important role in both nutrition and health protection for consumers of milk [1, 2]. Some epidemiological studies have clearly shown that raw milk can be infected with several pathogens, some of which are associated with human disease [3]. Brucellosis is a classic example of a zoonotic milk-borne disease caused by Brucella species that cause systemic disease and can also be found in mammary gland tissue and associated lymph nodes and shed in the milk in large numbers [4]. The disease is considered an occupational threat and is transmitted to humans through close contact with livestock, exposure to aborted materials and the ingestion of raw dairy products [5]. In Egypt, brucellosis is an endemic disease among animals and humans, Brucella melitensis biovar3 and Brucella abortus biovar1 are the main isolated species [6]. The infection in man occurs by two methods, one is wound infection and the other one is the ingestion [7]. The organism can survive in naturally contaminated unpasteurized milk for up to five days when kept at 4°C [8].

The routine identification and differentiation of Brucella species that based on culture and phenotypic traits are associated with a high risk of laboratory-acquired infections and is very time consuming also require biosafety level 3 laboratories [9, 10]. So, nowadays diagnosis of Brucella is mostly based on PCR techniques [11]. The qRT-PCR was used for the detection and diagnosis of different Brucella species [12, 13]. Moreover, some virulence genes (such as Ure, bvfA, and virB) specific to Brucella species were detected by [14]. BvfA gene (brucella virulence factor antigen) plays a role in the intracellular establishment, virB gene (type IV secretion system) is responsible for the translocation of virulence factors into mammalian cells and Ure gene (urease) protects Brucella from low pH [14].

This study aimed to detect Brucella organisms in Egyptian raw milk using cultural and molecular techniques.

2. Materials and methods

This study was conducted after under the ethical approval from the Experimental Animals Care Committee in compliance with the guidelines of Kafrelsheikh University.
2.1. Sampling
A total of 200 samples of raw milk were collected from bulk milk tanks located in El-Santa city, El-Gharbia governorate, and Sidi Salem city, Kafer El-Sheikh governorate (100 samples from each city). Samples were collected from collection centers all over one year in sterile falcon tubes and were transferred as soon as possible in an icebox to the laboratory with a minimum of delay to be examined for Brucella species. Each sample (30 ml) was mixed by inverting the falcon tube three to four times. The first 20 ml was used for bacteriological examination and the second 10 ml was utilized for DNA extraction. Milk samples used for bacteriological examination were stored at 4°C while samples for PCR assays were stored at -20°C.

2.2. Reagents
Bacto Brucella agar (Catalogue no. DFO 96401-3, Difco Laboratories, Detroit, Mich, USA) and a freeze-dried vial of selective antibiotic supplement (Catalogue no. SR 83, Oxoid Limited, Basingstokes, Hampshire RG24 OPW, UK) were used for isolation of Brucella. Monospecific anti Brucella abortus (A) and anti Brucella melitensis (M) sera obtained from central veterinary laboratory, New HAW, Weybridge, England were used for serotyping. Lyophilized reference Brucella strains [Brucella abortus biovar1 (strain 19) and Brucella melitensis biovar3] were obtained from Central Veterinary Laboratory New HAW, Weybridge, England. Power Prep™ DNA Extraction Kit was purchased (Reference no.E0101, Kogene Biotec). Gene JET Genomic DNA Purification Kit was purchased from Thermo Scientific™, USA, Cat. No. K0722. Primer design™ Ltd Brucella genus (all species) L-glutamine: 2-deoxy- scylloinosose amino transferase genesig® Advanced Kit, Serial no. JN68610-21593 with Oasig™ Lyophilised or Precision PLUS™ 2x qPCR Master mix. 5x FIREPOL master mix ready to load with 12.5mM mgcl2, 1 ml (Solis BioDyns Cat. No. 04-12-00125) was used.

2.3. Identification and serotyping of isolated cultures
Isolated cultures were identified and serotyped according to Alton et al. [15].

3.4. Detection of brucella by qRT-PCR
The extraction of bacterial DNA was performed according to the supplier manual using Kogene biotec kit for DNA extraction from milk samples and Gene JET Genomic DNA Purification Kit for DNA extraction from isolates. Brucella universal gene was amplified by qRT-PCR, using Primer design™ Ltd Brucella genus (all species) genesig® Advanced Kit, Serial no. JN68610-21593 with Oasig™ Lyophilised or Precision PLUS™ 2x qPCR Master mix. 5x FIREPOL master mix ready to load with 12.5mM mgcl2, 1 ml (Solis BioDyns Cat. No. 04-12-00125) was used.

2.7. Statistical analysis
Data were statistically analyzed by using SPSS software, computed and represented in tables.
3. Results and discussion

Brucellosis is mainly a disease of animals, while humans get infected through contact with infected animals or consumption of raw animal products especially dairy products made from unpasteurized milk. It is one of the most widespread zoonosis worldwide [17]. The Nile Delta region in Egypt is reported to have the highest human and animal densities in the world [18]. In Egypt, 62.1% of brucellosis cases were reported to people consumed Kareish cheese made from raw milk [19]. In the present study, 5 (2 samples from El-Santa city and 3 samples from Sidi-Salem city) out of 200 (with an incidence rate of 2.5%) milk samples were positive for Brucella and grow on Brucella specific media (Table 3). These colonies appeared round with smooth margins, translucent and of golden color (pale honey colored) (Figure 1). RT-PCR showed 43 positive samples with an incidence rate of 21.5% (10.5% and 11% from El-Santa city and Sidi-Salem city respectively).

Table 3. Incidence of Brucella in milk samples (n=200)

<table>
<thead>
<tr>
<th>Source</th>
<th>Culture method No.</th>
<th>%</th>
<th>RT-PCR No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>El-Santa city</td>
<td>2</td>
<td>1</td>
<td>21</td>
<td>10.5</td>
</tr>
<tr>
<td>Sidi-Salem city</td>
<td>3</td>
<td>1.5</td>
<td>22</td>
<td>11</td>
</tr>
<tr>
<td>Total positive</td>
<td>5</td>
<td>2.5</td>
<td>43</td>
<td>21.5</td>
</tr>
</tbody>
</table>

*Percent to total samples.

All colonies were identified biochemically, and with monospecific antisera as *Brucella melitensis*. The results were confirmed by using multiplex PCR with *Brucella abortus* and *Brucella melitensis* specific primers (Figure 2 and Table 4). All isolates were identified at product length 731 bp for *Brucella melitensis* (Figure 2).

Table 4. Biochemical, serological tests and multiplex PCR for identification of Brucella isolates

<table>
<thead>
<tr>
<th>Isolates NO</th>
<th>Co2 requirement</th>
<th>H2S test</th>
<th>Urease test</th>
<th>Catalase test</th>
<th>Agglutination with mono-specific antisera</th>
<th>Multiplex PCR for IS711 gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A: <em>Brucella abortus</em></td>
<td>M: <em>Brucella melitensis</em></td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>498bp</td>
<td>731bp</td>
</tr>
</tbody>
</table>

A: *Brucella abortus* antisera. M: *Brucella melitensis* antisera

Figure 1. Colonies on Brucella agar media appeared round with smooth margins, round edges, translucent and of golden color (pale honey colored, arrow).

Table 5. Incidence of Brucella in milk samples regarding seasons and sites (n=200)

<table>
<thead>
<tr>
<th>Positive samples by different tests</th>
<th>Culture method No.</th>
<th>%</th>
<th>RT-PCR No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summer</td>
<td>0</td>
<td>0</td>
<td>13</td>
<td>6.5</td>
</tr>
<tr>
<td>El-Santa city</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Sidi_Salem city</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>3.5</td>
</tr>
<tr>
<td>Winter</td>
<td>5</td>
<td>2.5</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>El-Santa city</td>
<td>2</td>
<td>1</td>
<td>15</td>
<td>7.5</td>
</tr>
<tr>
<td>Sidi_Salem city</td>
<td>3</td>
<td>1.5</td>
<td>15</td>
<td>7.5</td>
</tr>
</tbody>
</table>

*Percent to total samples.

These variations were declared by Corbel [24] who reported that in countries with low temperature or cold climates there is a marked seasonal variation in the incidence of acute brucellosis, with most cases occurring in the spring and summer where the period for abortions and parturitions among farm animals while the peak period in the winter.

The positive samples in summer and winter seasons were 0 and 5 by culturing while 13 and 30 by RT-PCR, respectively (Table 6). The lower recovery by culture was explained by Aulakh et al. [25] who attributed this to inactive state of infected animals of shedding organisms in milk, or due to physical and chemical management of milk before reaching to the consumers. High positive milk samples by qRT-PCR when compared to bacteriological culture method could be attributed to the very low numbers of bacteria, which would be consistent with the small number of colony forming units detected in milk before reaching to the consumers.
milk samples by culture methods [26] and because PCR can detect living and dead organisms, while culture detects only living organisms [27]. In addition, the culture technique is time consuming and presents a great threat of infection for the laboratory personnel [28].

Table 6. Sensitivity and specificity of different tests in relation to RT-PCR as gold standard (n=200)

<table>
<thead>
<tr>
<th>Test</th>
<th>Culture method</th>
<th>RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TP-5</td>
<td>FP-0</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>FN-38</td>
<td>TN-157</td>
</tr>
<tr>
<td>12%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Specificity</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>TP: true positive; FP: false positive; FN: false negative</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Of 200 milk samples examined 43 (21.5%) contained Brucella species as detected by qRT-PCR (Figure 3). Of them, 34 (79%), contained bvfA virulence gene (1282 bp), and 32 (74%) contained virB virulence gene (881bp) and 38 (88%) contained Ure virulence gene (2100bp) (Figures 4-6 and Table 7). Furthermore, all 5 Brucella isolates contained Ure and virB virulence genes and 4 of them contained bvfA gene (Table 7).
The Ure gene is the most virulence factor for *Brucella melitensis* as it responsible for urease activity which plays a role in the resistance of *Brucella melitensis* to low pH conditions consequently protect *Brucella melitensis* through the passage from the stomach [14]. Furthermore, some isolates, such as *Brucella abortus* are urease negative, while they seem to retain most of their pathogenic potential [29]. Whole-genome sequencing revealed the presence of a second urease operon (ureC2) with all the genes potentially active in *Brucella suis* and *Brucella melitensis*. On the other hand, *Brucella abortus* had two frame shift deletions in ureE2, and *Brucella ovis* had deletions in ureG2 and ureT [29]. Minnejad et al. [30] showed that *Ure* gene more frequently existed in *Brucella melitensis* strains. Because of the critical role of urease in pathogenesis, it has been hypothesized that *Brucella melitensis* is more virulent than *Brucella abortus*. So, each step in the Brucella infection process can be mediated by a number of virulence factors and each strain may have a unique combination of these factors that affected the rate of bacterial pathogenesis.

T4SS of Brucella encoded by the *virB* operon is a major virulence factor for *Brucella* [31]. Gorvel [32] and Celli et al. [33] reported that A mutant of the *VirB* type IV secretion system, which is necessary for intracellular survival, was unable to sustain interactions and fuse with the endoplasmic reticulum, and was killed via eventual fusion with lysosomes. Delrue et al. [34] stated that the type IV secretion system has been described in both *Brucella melitensis* and *Brucella abortus*. *Brucella* virulence factor A (*bvfA*) is a small 11 kDa periplasmic protein unique to the genus *Brucella* with no homologs in GenBank. Although *bvfA* was essential for *brucella* virulence, it may play a role in the establishment of the intracellular replication [35]. Derakhshandeh et al. [14] was in agreement with our study as, of 42 *Brucella melitensis* isolates; 33 (78.5%) isolates had *bvfA* genes, 31 (73.8%) isolates had *virB* genes and 37 (88.0%) isolates had *ure* genes.

### References


11. Navarro E, Segura JC, Castano MI. Use of real-time quantitative polymerase chain reaction to monitor the evolution of *brucella melitensis* DNA load during therapy and post therapy follow up in patients with brucellosis. Clinical Infectious Diseases 2006; 42: 1266-1273.


