Reliable and highly specific techniques for the detection of *Brucella* spp. antibodies in camel milk

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**Abstract**

Brucellosis is one of the most serious zoonotic diseases in human and farm animals caused by *Brucella* species. This study aims to: (i) estimate the current prevalence of *Brucella* among camels in Erbil Governorate; (ii) evaluate the milk ring test as a diagnostic tool for screening of brucellosis in camels; (iii) study the association between months and percentage of positive samples to *Brucella*. During the period, January - June 2021, a total of 250 raw camel milk samples (130 samples from farms and 120 from sale points) were randomly collected. The brucellosis is diagnosed using the Milk Ring Test (MRT), indirect ELISA, and bacteriological isolation of *Brucella* species. The prevalence of *Brucella* antibodies in camel milk samples is 11.6% and 10.4% according to MRT and ELISA, respectively. The overall isolation percentage of *Brucella* species is 8.4%. The detection rate of isolates in sale points is higher 10.0% than the isolation rate from farm 6.9%. The results also reveal that 4.6% and 5.8% are *Brucella abortus*; while, 5.8% and 4.2% are *Brucella melitensis* from the milk of farm and sale points, respectively. The highest rate of brucellosis according to MRT is observed in February 18.6%, while the lowest rate is documented in May 7.5%. We recommend using MRT for the diagnosis of *Brucella* spp. in routine screening of brucellosis in milk collection centers, dairy factories, and farm. Customers are also recommended to heat the milk adequately to eliminate this milk-borne pathogen before drinking milk or manufacturing processes.

**Keywords**: *Brucella abortus*, *Brucella melitensis*, ELISA, Erbil Governorate, MRT

**Introduction**

*Brucella* species are facultative intracellular, non-motile, non-sporing, gram-negative coccobacilli. They are aerobic, but some strains require 5-10% CO₂ for primary isolation. Growth in vitro is slow and primary isolation may require up to 4 weeks of incubation at 37°C. In vitro growth is slow but enhanced if serum was added to the growth medium (1). Brucellosis is a global bacterial zoonotic disease that is transmissible to humans and a wide range of domestic and wild animals, particularly food-producing animals comprising camels, cattle, buffaloes, sheep, goats, pigs, and reindeer (1,2). Camels in all rearing countries, except Australia, are infected by the two predominant species of the genus: *B. abortus* and *B. melitensis*. Brucellosis in camels is an insidious disease, since it hardly provokes any clinical signs, and may furthermore be faced with difficulties in laboratory diagnosis due to the lack of sufficiently validated tests (3). The prevalence of infection ranges widely between regions (4). For instance, the documented prevalence in East African countries range between <1% to 40%, while sporadic small-sized studies in the Middle East showed a prevalence of < 30% (5,6). Despite the massive rearing and exploitation of camels in many populations’ daily life in the Middle East, little is known about the true prevalence of brucellosis in the countries of this region. The presence of *Brucella* species in milk or dairy products can occur from either a direct passage from udders, contamination by animal...
excreta, or unsanitary handling of the milking utensils (4,7). The transmission to human occurs through the consumption of contaminated food of animal origin (milk or meat), and from mothers to breastfed babies (8,9). A meta-analysis study of Brucella spp. in raw milk has found the prevalence in the Middle East to be 29.0% (95% CI: 23-35%), which poses a serious threat to public health (6). In fact, human brucellosis is a multisystem disease with a broad spectrum of clinical manifestations and life-threatening complications, such as meningitis, osteomyelitis, sacroilitis, spondylitis, hepatic abscesses, peripheral arthritis, bronchopneumonia, epididymitis, prostatitis, orchitis, encephalitis, and cardiovascular complications (10,11). The diagnosis of brucellosis is rather complicated, and it must be obligatorily confirmed by laboratory testing. Bacteriological culture is still the definitive test for Brucella infections; however, various serological and molecular diagnostic tests (Rose Bengal test, ELISA, complement fixation, and slide agglutination test) are available with different accuracies and performance requirements (12-14). The choice of the diagnostic test depends on the overall epidemiological situation in the region, objectives of the study, validation of the diagnosis, monitoring, cross-sectional studies or confirmation of brucellosis free status of the region (15,16).

Different studies had found the sensitivity and specificity of MRT to range from 80-88% and 97-99% in the detection of brucellosis in milk samples from cows, sheep, and goats (17-19). However, another recent study found a lower sensitivity and specificity (73.3% and 84.6%, respectively) for the detection of brucellosis in milk samples from goats and sheep (20). This variation is mostly attributed to differences in disease prevalence and the accuracy of the reference test used for the evaluation of the diagnostic test (21). Nonetheless, and practically speaking, the adoption of MRT is supported by the observed high specificity of results as a straightforward and cheap screening/diagnostic method for excluding brucellosis rather than confirming the infection in the suspected animal. Recently, brucellosis in ewes, nanny goats, cows, and buffaloes in Erbil Governorate has been screened (17,18,22). However, to the best of the author’s knowledge, Brucella infection in the camel’s population has not been thoroughly addressed.

The objectives of this study are to: (i) estimate the current prevalence of Brucella among camels in Erbil Governorate; (ii) evaluate the milk ring test as a diagnostic tool for screening of brucellosis in camels; (iii) study the association between months (sample collection time) and frequency of Brucella.

**Materials and methods**

**Ethical approve**

This study obtained approval from the scientific board, College of Science, Knowledge University, Erbil, Iraq, the approval issue 001, dated 10/11/2020.

**Samples collection**

During the period, January - June 2021, a total of 250 raw camel milk samples (130 from farms and 120 samples from sale points) were randomly collected from suburban farms at the outskirts of Erbil city and retail milk shops in Erbil Governorate. For each sample, about 100 ml of milk sample were collected into labelled sterile plastic containers with screw lid, under hygienic conditions. On the same day of collection, all the samples were transported under cool conditions (inside an icebox ~ 5°C) to Department of Medical Laboratory Sciences at College of Science, Knowledge University, Erbil, Iraq. The samples were stored in a deep freezer at ~18°C and were analyzed within 48 hours of collection (23).

**Milk Ring Test (MRT)**

Detection of Brucella antibodies in raw milk was done by the Milk Ring Test, MRT. The test was carried out by adding one drop (~ 0.05 ml) of MRT antigen solution (JOVAC Jordan) to 1 ml of whole milk in a narrow test tube (11*100 mm). The antigen milk mixture was incubated at 37°C for 1-3 hours. If the anti-Brucella antibodies are present in the milk, they bind to the antigen and rise with the cream layer to form a blue ring above the white milk column. If antibodies are absent, the mixture remains homogeneously bluish white throughout the tube (19). The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the MRT were calculated according to standard equations below (21), using the bacterial isolation diagnostic method as the gold standard. Sensitivity = [TP / (TP + FN)] ×100. Specificity = [TN / (FP + TN)] ×100. Positive predictive value = [TP / (TP + FP)] ×100. Negative predictive value = [TN / (FN + TN)] ×100. Accuracy = [TP + TN / (TP + FP + TN + FN)] ×100. Where TP is the number of true positive, FP is the number of false positive, TN is the number of true negative, and FN is the number of false negative.

**ELISA test**

The indirect ELISA test to detect IgG were performed according to a published protocol (24). Briefly, all reagents were allowed to come to room temperature and homogenized by vortex before use. Samples were centrifuged to separate the creamy portion from the lactoserum containing the antibodies. Lactoserum was processed and tested according to the instructions provided by the kit manufacturer (Euroimmun AG, Germany).

**Isolation and identification of Brucella**

Isolation of Brucella species from the raw milk samples was performed under aseptic conditions (25). Inoculated tubes and plates (Brucella broth and Brucella agar, HiMedia, India) were incubated aerobically and in the presence of 5%-10% carbon dioxide at 37°C. The plates were observed for up to 7 days for the presence of suspected colonies of
Brucella. Biochemical tests were performed for identification purposes of the suspected isolates (17). The identification of B. abortus and B. melitensis were confirmed by its definitive biochemical tests (1).

Statistical analysis
Data were analyzed using SPSS software version 25, and confidence intervals were estimated using normal distribution approximation at an alpha level of 0.05. Chi-square test was applied to test the odd between the groups. P value less than 0.05 was considered significant. Association of seroprevalence with months was evaluated using correlation coefficient test.

Results

Seroprevalence of Brucella
According to the MRT, the overall prevalence of Brucella antibodies in raw milk samples was 11.6% (29/250). Similarly, 10.4% (26/250) of milk samples were positive for the presence of anti-Brucella antibodies, detected by ELISA (Table 1). Based on both tests, there is no significant difference between raw milk collection sites in terms of positivity rate (χ² = 0.416). Statistically, it is estimated that 7.91-16.23% (95% CI) of camels would be seropositive for Brucella in Erbil Governorate if screened by MRT assay. No significant differences were found between MRT and ELISA in terms of brucellosis detection (χ² = 0.183, P=0.668).

Prevalence of Brucella species
The overall isolation percentage of Brucella species from camel raw milk samples was 8.4% (21/250). It is obviously clear that the detection rate in sale points was higher 10.0% than the isolation rate from a farm 6.9%. However, such an increase is not statistically different (p=0.378). Regarding the identified species of Brucella from camel raw milk samples, B. abortus comprised about two thirds 61.9% of total isolates (13/21 isolates), while the remaining isolates were of B. melitensis (Table 2).

Table 1: Prevalence of Brucella antibodies in camel raw milk

<table>
<thead>
<tr>
<th>Collection Site</th>
<th>Samples</th>
<th>Positive Samples n (%)</th>
<th>95% CI</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRT: Farm</td>
<td>130</td>
<td>13 (10.0)</td>
<td>5.43 - 16.49</td>
<td>0.416</td>
</tr>
<tr>
<td>Sales points</td>
<td>120</td>
<td>16 (13.3)</td>
<td>7.82 - 20.75</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>250</td>
<td>29 (11.6)</td>
<td>7.91-16.23</td>
<td></td>
</tr>
<tr>
<td>ELISA: Farm</td>
<td>130</td>
<td>11 (8.5)</td>
<td>4.30 - 14.64</td>
<td>0.302</td>
</tr>
<tr>
<td>Sales points</td>
<td>120</td>
<td>15 (12.5)</td>
<td>7.17 - 19.78</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>250</td>
<td>26 (10.4)</td>
<td>6.91 - 14.87</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Isolation of Brucella species from camel raw milk

<table>
<thead>
<tr>
<th>Collection site</th>
<th>No.</th>
<th>B. abortus n (%)</th>
<th>B. melitensis n (%)</th>
<th>Total n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farms</td>
<td>130</td>
<td>6 (4.6)</td>
<td>3 (2.3)</td>
<td>9 (6.9)</td>
</tr>
<tr>
<td>Sale points</td>
<td>120</td>
<td>7 (5.8)</td>
<td>5 (4.2)</td>
<td>12 (10.0)</td>
</tr>
<tr>
<td>Total</td>
<td>250</td>
<td>13 (5.2)</td>
<td>8 (3.2)</td>
<td>21 (8.4)</td>
</tr>
</tbody>
</table>

Comparison of MRT and ELISA to culture approach
The MRT technique detected more cases of brucellosis 11.6% than the traditional culture method 8.4% in both groups of milk samples. Sensitivity, specificity, positive predictive value, and negative predictive value of MRT and ELISA are given in (Table 3).

Temporal distribution of seropositive samples
Variations of Brucella antibodies occurrence in camel raw milk samples during six months have been investigated (Figure 1). The highest rate of prevalence of Brucella antibodies detected by MRT was found in February 18.6%, while the lowest rate was documented in May 7.5%. According to the statistical calculations, there is a weak positive correlation (r² = 0.16) between the progress of winter-spring months and the prevalence of brucellosis.

Discussion
Brucellosis is an infectious disease of livestock and wild animals and the commonest human zoonosis. Transmission to humans occurs in several ways, commonly through consumption of contaminated food, particularly raw milk or meat and their products (26-28). Our findings are inconsistent with a previous country-wide survey of the camel brucellosis in Iraq that was carried out in 2005, in cooperation with FAO, in which no antibodies were detected against Brucella in 540 serum samples screened using the Rose Bengal and ELISA tests (29). Indeed, prevalence of
camel brucellosis was reported in nearby countries such as Saudi Arabia, where the seroprevalence of brucellosis in camels appeared to follow two distinct patterns: a low (2-5%) prevalence in nomadic or extensively kept camels and a high 8-15% prevalence in camels kept intensively or semi-intensively (5). Moreover, 12.1% of camels were found infected with Brucella in Jordan (30). These findings indicate a current increasing trend of brucellosis in camel population in Erbil.

The isolation of Brucella from milk samples may be improved if more than one culture medium is used (12,13). On the other hand, higher isolation rates were also reported from different countries. In Syria, a recent study isolated Brucella melitensis from bovine raw milk samples at a rate of 25% (39). Furthermore, in San Paulo, 30% of bovine screened milk samples yielded Brucella abortus during a study of four years (40).

MRT primarily detects IgA and IgM antibodies against Brucella spp. in raw milk. The sensitivity and specificity of MRT is reported to being 85% and 95%, respectively (17,41). The sensitivity and specificity of MRT reported in this work clearly prove its good value as a straightforward, inexpensive screening test to detect brucellosis in raw milk of cattle and buffaloes. However, a higher sensitivity of 100% and lower specificity of 75-73.5% have been reported for the MRT testing of cow and buffalo milk samples (42). It should be noted that the slight drawback of MRT specificity in comparison to molecular diagnostic techniques is compensated by the fact that the MRT is cheap and easy to perform. Meanwhile, ELISA and PCR approaches are expensive and unavailable in many developing countries. Albeit, a recent Syrian study has found that PCR and culture approach yielded the same results, while the MRT showed lower rates of positive results (39).

The temporal distribution of seropositive raw milk samples of camel shows a poor correlation between months and the prevalence of brucellosis. The seasonality of brucellosis in camel is still largely unknown. The wet season has been reported to be a risk factor for the infection (43,44). It is believed that Brucella spp. do not survive in the dry and warm weather (43). It is worthwhile to say that camels appear to be infected by spill-over of Brucella from other ruminants and cattle (5,43). In such cases, camels in cohabitation with other mammals are exposed continuously to Brucella regardless of the season and may show such irregular seasonality. Nonetheless, the final genotypic solid evidence supporting this observation is still missing.

Conclusion

The rate of brucellosis in camel milk at Erbil Governorate is alarming to the risk for humans. MRT can be used for efficient and everyday monitoring due to its simple, rapid, sensitive, and cheap technique for routine screening of brucellosis in raw milk. The epidemiology and seasonal variations in brucellosis rates in camel at Erbil are not completely clear. Further research addressing this subject are greatly recommended.

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Conflict of interest

There is no conflict of interest.

References


تكتني تقنية موثوقة ودقيقة لكشف عن الأجسام المضادة لبكتيريا البروسيلا في حليب الحمام

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الخلاصة

يعد داء البروسيلا واحداً من أهم الأمراض البكتيرية المنقولة من حيوانات المزارع إلى الإنسان. وتحدد هذه الدراسة لتقييم الانتشار الحالي لمرض البروسيلا بين الحمام في محافظة أربيل. وتقييم اختبار الحلقة للحليب كفحص تشخيصي للكشف عن داء البروسيلا في الحمام. كما يتطرق البحث لانتشار البروسيلا خلال فترة الدراسة. جمعت عينة حليب خام عشوائياً من 350 عينة من حمام المزارع، بينما 100 عينة من نقاط بيع الحليب خلال الفترة الزمنية من 2022.7.29 إلى 2023.6.30. شُخص المرض باختبار الحلقة للحليب، والمقاومة المناعية للإنزيمات المرتبطة بالحليب، والتحليل الاحصائي. وترى النتائج وجود الأجسام المضادة في 11.6 و10.4% من العينات من المزارع، وذلك بالاختبارات. أما بالنسبة للأنواع البكتيرية، شكل النوع بروسيلا إليتونسي نسبة 4.6 و5.8% من العينات من المزارع، وعينة نقطية للبيضة على التوالي. بينما شكل النوع بروسيلا ديوتينسيdesi نسبة 4.2% من عينات المزارع وعينات نقطية للبيضة على التوالي. وفيما يخص الانتشار généralisé، فإن أعلى نسبة سجلت في شهر شباط بنسبة 18.6%. ويبدو على هذه النتائج، فإنه يمكن الاعتماد على اختبار الحلقة للحليب في الكشف السريع عن داء البروسيلا في المزارع والمصانع ونقاط البيضة. كما ننصح المستخدمين بتسخين الحليب بشكل كافٍ قبل تناوله أو استخدامه في تصنيع منتجات الحليب وذلك لتفادي الإصابة بالبروسيلا.