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Serological and bacteriological evaluation of brucellosis in milk of small ruminants

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Abstract

Brucellosis continues to be a serious infection to human and animal populations in developing countries with detrimental impacts on public health and food animal production. This work aimed to estimate the prevalence of brucellosis in sheep and goats' raw milk samples at Kirkuk Governorate, Iraq by identifying anti-*Brucella* antibodies and isolation of *Brucella* species. A total of 430 raw milk samples (210 sheep milk and 220 goats' milk) were randomly collected from dairy females during the period from July to December 2019. The results showed an overall prevalence of *Brucella* antibodies in 12.3% and 10.7% of animals according to MRT and indirect ELISA, respectively. The overall isolation of *Brucella* species from raw milk samples was 10.0%. The isolated species of *Brucella* were *B. abortus* (37.2%) and *B. melitensis* (62.8%). An observable increase in occurrence during autumn (September to November) was detected, while autumn progress was associated with declining in brucellosis. In conclusion, brucellosis is still a significant public health hazard in Kirkuk Governorate. Based on test performance, the study recommends MRT as a rapid screening test for detecting brucellosis in milk in farms, centres, and dairy factories. Consumers are also recommended to sufficiently pasteurize the milk in order to kill this milk-borne pathogen before consumption.

1. Introduction

Brucellosis is a zoonotic disease worldwide with a remarkably higher prevalence in Mediterranean Basin countries, the Middle East (including Iraq and Iraqi Kurdistan and the Arabian Gulf), and the Indian subcontinent. Although most European countries, United Kingdom, Australia, Canada, and Japan were declared brucellosis-free, other countries in Asia, Africa, and Central and South Americas are still reporting brucellosis, nonetheless, the infection is sometimes unrecognized on certain occasions (Wareth *et al*., 2014; Hull and Schumaker, 2018; Dafale *et al*., 2020).

Brucellosis can be caused by four different *Brucella* species in humans; *B. abortus*, *B. melitensis, B. suis*, and *B. canis.* Among these, *B. melitensis* is the greatest virulent followed by *B. suis*. As few as 10 to 100 organisms can cause the disease in humans (Bukhari, 2018; El-Sayed and Awad, 2018; Nejad *et al*., 2020). In sheep and goats, brucellosis is mainly caused by *B. melitensis* which contains three biovars named biovars 1, 2 and 3. The distribution of *B. melitensis* has long been associated with the Mediterranean littoral, however, it is now known to be much more widely distributed in South- East Asia, North Europe, and North America (Franc *et al*., 2018; Tekle *et al*., 2019). Microbiologically, *Brucella* species are intracellular, non-motile non-sporing, gram-negative coccobacilli. They are aerobic, but some strains require 5 -10% CO_2 for primary isolation and may require up to 4 weeks of incubation at 37°C. Growth in vitro is slow and primary isolation but improved by the addition of serum or blood (Araj, 2015; Tille, 2017).

Human brucellosis is a foodborne and professional zoonotic disease contracted by unpasteurized milk or other dairy products and undercooked meat. The portal of entry is either the mucous membranes or abraded skin. Exposure to contaminated material during helping at birth and inhalation were also reported as potential routes (Singh *et al*., 2018; Jara *et al*., 2020). In the intestines, brucellae are phagocytosed by lymphoepithelial cells of gut-associated lymphoid tissue, from which they gain access to the submucosa. However, brucellae are quickly ingested by polymorphonuclear leukocytes, which usually fail to kill them, and are also phagocytosed by macrophages. Bacteria transported in macrophages,

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which travel to lymphoid tissue draining the infection site, may finally localize in mammary glands, liver, spleen, lymph nodes, joints, bone marrow and kidneys (Boschiroli *et al*., 2002; de Figueiredo *et al*., 2015).

Reproductive organs are also reached and play a key role in the transmission of *Brucella* during breeding seasons. As a result of heavy colonization (≈ 10^9 CFU/ gm), placenta and fetal fluids are the most important route for transmission in abortion events. In addition, sheep and goats shed the bacteria for long periods through milk, urine, and mucosal secretions. In addition to its clinical significance and public health hazard, brucellosis is the major cause of direct financial losses due to major drawbacks in the international trade of milk, meat, and their products (de Figueiredo *et al*., 2015; Singh *et al*., 2018; Jara *et al*., 2020).

Accurate diagnosis of brucellosis is reached by a combination of testing essays owing to the lack of a single definitive test. Different diagnostic methods are available for various purposes; screening, confirmatory, and surveillance (Godfroid *et al*., 2010). Milk ring test (MRT) is an indirect assay for the detection of anti-*Brucella* antibodies in milk using *Brucella* whole-cell antigen. In contrast, bacteriological isolation is more accurate than MRT (Musallam *et al*., 2015). In Kirkuk Governorate, livestock brucellosis is not addressed sufficiently, hence, this study aimed at investigating the prevalence of brucellosis by serological and bacteriological isolation of *Brucella* from raw milk sold in retail markets in Kirkuk Governorate between July and December 2019.

2. Materials and methods

2.1 Study design and sampling of milk

A total of 430 raw milk samples were collected under aseptic conditions during the period from July to December 2019. Study milk samples were 210 from sheep and 220 from goats sold in Kirkuk Governorate. The samples were collected according to (Almashhadany and Osman, 2019) and transported in a Coleman cooler for less than an hour to the Microbiology Laboratory in the Department of Medical Laboratory Sciences (Knowledge University, Iraq).

2.2 Detection of Brucella antibodies

2.2.1 Milk Ring Test (MRT)

In the laboratory, the detection of *Brucella* antibodies in milk was done by Milk Ring Test (MRT) antigen solution kit (JOVAC Jordan). The test was carried out according to previously published literature (Almashhadany, 2019). Briefly, one drop (0.03 mL) of hematoxylin-stained antigen is mixed with 1 mL of milk in a narrow test tube (11×100 mm) and incubate at 37˚C for 1 – 3 hrs. If the specific antibody is present in the milk it will bind to the antigen and rise with the cream to form a blue ring at the top of the milk column (Ring white and column blue indicate a negative result).

2.2.2 ELISA

The indirect ELISA tests were performed according to the manufacturer's protocol (ID Screen® Brucellosis, ID Vet Innovative Diagnostics, France).

2.3 Bacteriological isolation and identification

The isolation of *Brucella* from milk was done under sterile conditions according to (Hadad *et al*., 1997; Al-mashhadany, 2018a). The identification of *B. abortus* and *B. melitensis* were confirmed by biochemical analysis performed according to a previously published scheme (Corbel, 2006; Al-mashhadany, 2018b).

2.4 Statistical analysis

Data were analysed using SPSS software version 15, confidence intervals were estimated using normal distribution approximation at an alpha level of 0.05. Chi-square test was applied to test the differences between groups. The sensitivity and specificity of the MRT were calculated according to standard equations, using the bacterial isolation method as a gold standard.

3. Results

3.1 Prevalence of Brucella antibodies according to MRT

The overall rates of *Brucella* antibodies in raw milk samples were 12.3% and 10.7% for MRT and ELISA respectively (Table 1). The proportion of positive samples among goat's milk was higher than found in sheep milk samples. Statistically, it is estimated that up to 15.81% (95% confidence interval) of goats and sheep milk would be seropositive for *Brucella* in Kirkuk Governorate if screened by MRT. No significant difference was noted between the detection rate of both tests (P = 0.462).

3.2 Isolation of Brucella species from milk

Depending on phenotypic properties two *Brucella* species were identified; *B. melitensis* and *B. abortus* in 43/430 (10.0%) samples. Most of the isolates were recovered from goat's milk (55.81%) while the remaining portion (44.18%) was from sheep milk (Figure 1). Generally, *B. melitensis* was significantly more prevalent than *B. abortus* (P < 0.005) in both raw milk types. Based on this sample, 7.51% - 13.20% of goats

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and sheep milk samples are expected to contain viable cells of brucellae (95% CI).

Table 1. Seroprevalence of *Brucella* antibodies among sheep and goats raw milk.

	Samples	Positive samples n (%)	95% CI	P value
MRT				
Sheep milk	210	24 (11.4)	7.46-16.53	0.571
Goats milk	220	29 (13.2)	9.01-18.38	
Total	**430**	**53 (12.3)**	**9.37-15.81**	
ELISA				
Sheep milk	210	20 (9.5)	5.91-14.33	0.441
Goats milk	220	26 (11.8)	7.87-16.84	
Total	**430**	**46 (10.7)**	**7.94-14.01**	

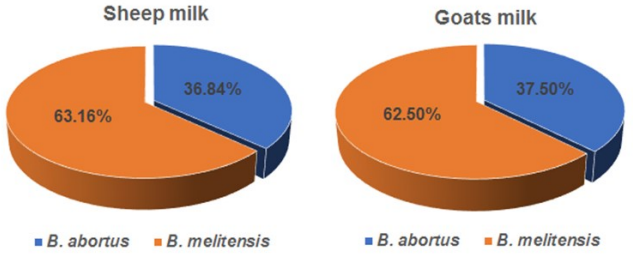

Figure 1. Distribution of isolated *Brucella* species in tested milk samples

3.3 Diagnostic performance of MRT and ELISA

The MRT technique detected more cases of brucellosis (12.3%) than the traditional culture method (10.0%) in both milk types. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of MRT are given below in Table 2. The efficiency of MRT in detecting brucellosis in screened ruminants is 95.9% in comparison to the culture method, which candidates the MRT to be a good alternative screening/diagnostic method.

Table 2. Diagnostic performance of MRT and ELISA in comparison to culture method

	MRT	ELISA
Sensitivity	81.48% (61.92-93.70)	86.96% (66.41-97.22)
Specificity	97.93% (94.78-98.43)	97.96% (94.86-99.44)
PPV	84.62% (67.23-93.65)	83.33% (65.17-93.04)
NPV	97.42% (94.48-98.82)	98.46% (95.70-99.46)
Accuracy	95.91% (92.38-98.11)	96.80% (93.53-98.71)

3.4 Seasonal variations of brucellosis

Variations of *Brucella* antibodies incidence in sheep and goats raw milk samples during the study have been noted to follow a circadian pattern (Figure 2). According to MRT, the highest rate of occurrence of *Brucella* antibodies was noticed in November (17.6%), while the lowest rate was recognized in August (7.2%).

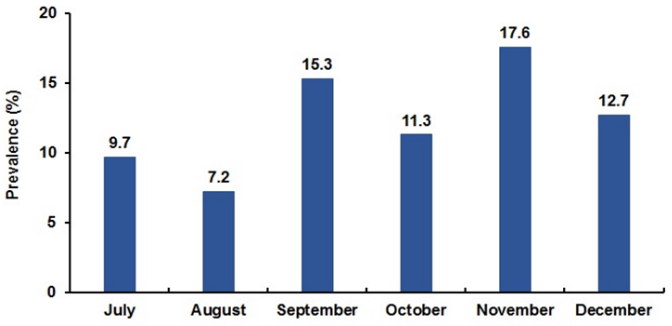

Figure 2. Seasonality of seropositive cases of brucellosis in goats and sheep milk samples.

4. Discussion

Brucellosis is an important infectious disease of livestock and wild animals and is also the commonest human zoonosis. Although being a primary disease of food-producing animals, it has the transmissibility to humans in several routes, commonly through contaminated food, mainly raw milk or meat and their products (Al-mashhadany, 2009a, 2009b; Almashhadany, 2014; CDC, 2020). Lactating females play a critical role in the epidemiology of brucellosis, because the *Brucella* species concentrate in the supra mammary lymph nodes and mammary glands in more than 80% of infected females, which persist to excrete *Brucella* in their milk for long times (Njuguna *et al*., 2017; Raghunandan *et al*., 2018).

Out of 430 sheep and goats milk samples, the overall rates of *Brucella* antibodies detection in raw milk samples were 12.3% and 10.7% according to MRT and ELISA, respectively (Table 1). These findings are in good agreement with a study conducted in Erbil city (Iraqi Kurdistan) where the overall occurrence of *Brucella* antibodies in ewes and nanny goats milk samples was 9.7% (Al-mashhadany, 2018b). They are also consistent with the findings reported from Al-Samawa city (Iraq) where MRT detected anti-*Brucella* antibodies in 9.16% of samples (Najum, 2014). Similar rates were also documented in Pakistan (9.4%) (Ali *et al*., 2018), Saudi Arabia (11% to 12%) (Abdellatif, 2019), and Sudan (9.3%) (Shuaib *et al*., 2018). On the other hand, higher rates were reported in Libya (21%) (Al-Griw *et al*., 2017), India (27.05%) (Dubey *et al*., 2017), Iran (45.5% and 27.3% of goats and sheep milk, respectively) (Moslemi *et al*., 2018), and Egypt (56.2% - 36.4%) (Ibrahim *et al*., 2012). These variations are mostly attributed to the difference in epidemiology, geographical locations, and the diagnostic tests employed. Moreover, ELISA-based screening of marketed milk samples in Duhok city (Iraqi Kurdistan) also showed a high rate (61.25%) (Turgay and Ahmed, 2016).

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Brucellosis in food-producing animals in Iraq has been tackled by only a few studies that reported prevalences of the disease in Iraq (including the Kurdistan region) ranged from <1% to 20% among various livestock animals (Omar, 2011; Al-Hussain and Thaer, 2012; Jaff, 2016; Alhamada *et al*., 2017; Al-mashhadany, 2018b; Almashhadany, 2019; Dahl, 2020). Recently, an Iraqi recent study isolated both *B. abortus* and *B. melitensis* from the milk of cows and buffaloes and found only 7.1% were culture-positive for *Brucella*. The percentage of isolated species were 70% and 60% for *B. abortus*, while 30% and 40% were *B. melitensis* from the milk of cows and buffaloes, respectively (Almashhadany, 2019). Additionally, our results are consistent with a study from Iran that found 9.6% of screened sheep milk samples (n = 125) harbouring *B. melitensis*, however, 18% of goat milk samples were positive for *B. melitensis* (Shakerian *et al*., 2016).

The bacteriological recovery of brucellae in milk usually shows lower rates than serological tests. It is usually difficult to recover *Brucella* from milk by culture owing to the impaired viability of brucellae cells outside animal hosts (Hull and Schumaker, 2018). In a large study in Egypt, the isolation of *Brucella* was successful only in 33.8% of samples where serodiagnosis and PCR approaches showed positivity in more than 40% of samples (Ibrahim *et al*., 2012). Similar observations with low isolation rates (<4%) were also documented in Iran (Ashrafganjooyi *et al*., 2017; Shirazi *et al*., 2017). Additionally, sampling different area with different *Brucella* epidemiology or during the dry season may account for such variations in isolation rates (Corbel, 2006). The isolation of *Brucella* from milk samples may be improved if more than one culture medium is used.

The diagnostic performance of MRT (Table 2) in comparison to the culture approach clearly reveals its good value as a straightforward, inexpensive screening test to detect brucellosis in raw milk of goats and sheep. However, a higher sensitivity of 93.1 and 93.02 was reported in the case of milk samples collected from sheep and goats, respectively (Ibrahim *et al*., 2012). It should be noted that MRT has a lower sensitivity in comparison to the culture method and current molecular diagnostic techniques, but this drawback is compensated by the fact that the MRT is cheap and easy to perform. The diagnosis of brucellosis is rather complicated and it has to be obligatorily confirmed by laboratory testing. The choice of the diagnostic tools depends on the overall epidemiological situation in the region and the objectives of the study; validation of the diagnosis, monitoring, cross-sectional studies or confirmation of the brucellosis-free status of the region (Smirnova *et al*., 2013).

The gradual decrease in the seropositive rates of brucellosis in raw milk during July and August could be attributed to the gradual increase in humidity and rain level in the Kurdistan region. Indeed, wet season (odds ratio 3.7, 95% CI 1.5–9.1) was found to be a risk factor for seropositive brucellosis in camel and goat populations (Megersa *et al*., 2010). This observation is also supported by other studies from Kenya (Maiyo and Obey, 2016) and Iran (Nematollahi *et al*., 2017; Riabi *et al*., 2017).

5. Conclusion

Brucellosis is a serious health concern and economically significant in different areas including the Kurdistan region. MRT can be used for fast routine monitoring of lactating sheep and goats because this test is a simple technique for day-to-day surveillance of Brucellosis in milk. The findings of this study indicated that the prevalence of *Brucella* antibodies among sheep and goats' milk in Kirkuk Governorate seems to be high and dangerous for public health. Consumers are recommended to properly heat raw milk to kill this milk-borne bacteria. Promotion of health awareness through the media (visual media, audio, and newspapers) is advised to highlight the method of transmission and prevention of animal and human brucellosis.

Conflict of interest

The author declare no conflict of interest.

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