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Diagnosis of brucellosis in sheep and goats raw milk by fast and reliable techniques

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Abstract

This study aimed to evaluate the occurrence of brucellosis in sheep and goats' raw milk samples by detecting anti-*Brucella* antibodies and *Brucella* species at Erbil governorate. A total of 320 raw milk samples (150 sheep milk and 170 goats milk) were irregularly collected from dairy females. The overall occurrence of Brucella antibodies in samples of sheep and goats raw milk was 11.6% and 9.7% according to MRT and indirect ELISA, respectively. Isolation of *Brucella* (*B.*) species from samples of raw milk was 7.8%. The isolated species of *Brucella* were *B. melitensis* and *B. abortus*. A noticeable increase in frequency during September to November was observed. In conclusion, brucellosis is still a considerable public health threat in the Erbil. Based on the tests performance, the study recommends MRT in standard observing of brucellosis in milk aggregate farms, centers, and dairy manufactories. Customers are also recommended to adequately pasteurize the milk in order to damage this milk-borne pathogen before ingestion or saleable handling.

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Introduction

Brucellosis is a severely weakening and deactivating classical bacterial zoonotic disease, it signifies a high public health threat and poses a major threat to human health. It is a very old zoonotic disease since animals are the only source of infection, and a recent indication from Egyptian ancient skeletons has revealed that brucellosis has been existing for no less than 750 BC. *Brucella* species can be traced back to 2.8 million years by probable indication of pathologic variations in a late Pliocene hominin skeleton. Furthermore, molecular tests demonstrated the continuance of *B. melitensis* DNA in a 700-year-old skeleton from medieval Italy, and confirming the permanence of this zoonotic contagion, and even a specific lineage, in the Mediterranean area over the centuries (1,2). According to the WHO, the FAO, and the Office International des Epizooties (OIE), brucellosis is yet one of the most significant and prevalent zoonoses globally, but also ranks as one of the seven record neglected diseases (NDs) according to the World Health Organization, due to its non-descript clinical presentation in human inhabitants. Brucellosis has been eradicated in many developed countries, but it is still prevalent in various regions, particularly in the Mediterranean Basin, Africa, the Middle East (including Iraq), the Arabian Gulf, the Indian subcontinent, and some developed countries with a low income, limited resources, and frequent contact with livestock animals. There are approximately 500.000 new cases of human brucellosis are reported yearly around the world, with around 10 per 100,000 populations. However, many researchers declared that this figure underestimates the realistic epidemiology and the actual occurrence is assessed to be 5,000,000 to 12,500,000 cases per annum (3). This disease is caused by the Gram-negative bacterial genus *Brucella*. Food sources of infection are typically the unpasteurized milk and dairy products, undercooked meat, animal bone marrow, and probably undercooked seafood. Human brucellosis is acquired essentially through direct touch of damaged skin or mucous membrane with infected animal tissues, consumption of infected meat or

unpasteurized dairy products, and inhalation of infectious aerosols. Although the Human-to-human transmission is still under debate, it has been described to occur through vertical and sexual routes. The transmission may also take place through blood transfusions or tissue transplantation, and exposure to contaminated material during helping at birth. Breastfeeding mothers infected with Brucella may pass the infection on to their babies (4). At the time, at least 12 *Brucella* species are documented under *Brucella* genus; *B. abortus*, *B. melitensis*, *B. ovis*, *B. canis*, *B. suis*, *B. neotomae*, *B. ceti*, *B. pinnipedialis*, *B. microti*, *B. Papionis*, *B. inopinata*, and *B. vulpis*. Species are differentiated by the production of urease and H_2S, dye sensitivity, cell wall antigens, and phage sensitivity. The major species are divided into multiple biovars. *Brucella* species are facultative intracellular, gram-negative coccobacilli (short rods) measuring about 0.6 to 1.5 µm by 0.5-0.7 µm, non-spore-forming, lack capsules or flagella, therefore, are non-motile (5). Growth Brucella species in vitro are slow and primary isolation may require up to 4 weeks of incubation at 37°C. They are aerobic, but some strains require 5-10% CO_2 for primary isolation. A handful of culture media was devised for the cultivation of *Brucella* such as *Brucella* agar, albumin agar, and trypticase soy agar that may be enriched by the addition of blood or serum. Also, there are four unnamed isolates without reported infections, *Brucella* NFXXXX Australian rat (6), *B.* unnamed Blue dotted ray; *B. inopinata*-like 09RB8471 African bullfrogs and Big-eyed tree frog, and *Brucella* UK8/14 White's tree frog. *Brucella* species can grow and survive for protracted periods in the environment, it remains viable outside animal hosts for the duration ranging from less than a day to > 8 months, depending on environmental factors such as temperature, humidity, exposure to sunlight, and the presence of organic matter. Indeed, survival is longer (months to years) when the temperature is low. In circumstances of low temperatures, high humidity, and no sunlight, *Brucella* species may remain viable for several months in aborted fetuses, manure, water, wool, hay, and other materials. They also can withstand drying, especially when organic material is present, and can survive in soil and dust. Their persistence in unpasteurized cheese is influenced by factors such as the temperature, water content, and type of fermentation, pH, and ripening time. Survival times of years have been reported in frozen meat (7).

Brucella species have a wide range of domestic, wild animals, and marine hosts, including cattle, buffaloes, camels, sheep, goats, pigs, reindeer, seals, cetaceans, and voles. In sheep and goats, brucellosis is mainly caused by *B. melitensis*, which contains three biovars; 1, 2, and 3. The distribution of *B. melitensis* has long been associated with the Mediterranean littoral regions, however, it is now known to be much more widely distributed with South- East Asia, North America, and North Europe (8).

Brucella infection is one of the major bacterial agents that cause tremendous economic losses due to abortion in sheep and goat flocks in many countries. In the livestock industry, the disease is manifested by abortions during the last third of pregnancy, stillbirths, weak lambs and kids, infertility and characterized essentially by epididymitis and orchitis in males, placentitis in pregnant female animals, with excretion of the organisms in milk and uterine discharges in female animals. It also causes a significant loss of productivity through high morbidity. Sheep and goats' brucellosis is prevalent globally, however, it is remains a serious threat among low- and middle-income countries (9).

Usually, it is difficult to diagnose *Brucella* because of the long incubation period, which ranges from 5 days to 5 months. The diseases can progress in different forms, acute, chronic, or asymptomatic, also the brucellosis has to be obligatorily confirmed by laboratory testing. The choice of diagnostic tools depends on the objectives of the study and on the general epidemiological situation of the disease in the region. Overall, rapid serological tests employing serum, milk, meat juice are the most straightforward and affordable, while bacteriological isolation and identification of *Brucella* is an exhausting process that might require weeks (10).

The epidemiology of *Brucella* among the food of animal origin at Erbil Governorate is not well established so far, therefore, the objectives of this study were to study the occurrence of brucellosis in Erbil governorate by detecting anti-*Brucella* antibodies and *Brucella* species in raw milk samples from sheep and goat by using MRT and indirect ELISA. The sensitivity and specificity of MRT and ELISA were evaluated according to standard equations; where the brucellosis isolation method was adopted as the gold standard. Also, the seasonal variations of brucellosis were addressed.

Materials and methods

Samples collection and transport

Throughout the interval from July to December 2019, a total of 320 raw milk samples from sheep and goats were obtained (150 from sheep and 170 samples from goats). Each sample (about 200 ml) was randomly collected aseptically into labeled sterile plastic containers from retail milk shops in different markets in Erbil Governorate. Under cool conditions, samples were transported directly to the Department of Medical Lab Science (DMLS) at Knowledge University (KNU). The samples were stored in deep freezer at -18°C and were evaluated within 48 - 72 hours of the collection (11).

Milk Ring Test (MRT)

Anti-*Brucella* antibodies were detected in raw milk samples by the milk ring test (MRT) antigen (JOVAC, Jordan), according to manufacturer's instructions. Briefly, one drop (~ 0.05 ml) of MRT reagent was added to 1 ml of whole milk in a narrow test tube (11 x 100 mm) and incubated at 37°C for 1-3 hours. In the absence of antibodies to anti-Brucella, the mixture remains homogeneously bluish

white through the tube. The presence of Anti-*Brucella* antibodies is inferred by a white mixture with a blue ring on the top (10).

Indirect ELISA test

The indirect ELISA tests were performed according to the manufacturer's protocol (ID Screen® Brucellosis, ID Vet Innovative Diagnostics, France). Briefly, all reagents were allowed to come to room temperature (21°C ± 5°C) before use and homogenized by inversion or vortex. Milk sample was allowed to still so that the cream separates from the lactoserum (cream on the top, lactoserum on the bottom). Lactoserum containing the antibodies was drawn carefully to a separated sterile clean tube for indirect ELISA reaction (12).

Isolation and identification of *Brucella*

The Brucella species were isolated from raw milk samples was done under sterilized conditions, according to the standard methods adopted in this field. Inoculated plates (*Brucella* broth and *Brucella* agar, HiMedia, India) were incubated aerobically, as well as in the existence of 5%-10% carbon dioxide at 37°C.The incubated plates were examined for up to 7 seven days. The questionable colonies were subjected to biochemical tests for identification purposes (10,12).

Diagnostic performance of MRT and indirect ELISA

The sensitivity and specificity of MRT and indirect ELISA were evaluated according to standard equations; where the brucellosis isolation method was adopted as the gold standard.

Statistical analysis

Data were analyzed using SPSS software version 25, confidence intervals were assessed using normal distribution approximation at an alpha level of 0.05. Chi-square test was applied to test of the difference between groups.

Results

Seroprevalence of *Brucella*

The overall occurrence of *Brucella* antibodies in raw milk samples was 11.6% according to MRT (Table 1).

The results showed that the percentage of positive samples among goat's milk was insignificantly higher than that found in sheep milk samples. It is assessed that up to 15.6% (95% CI) of the sheep and goats raw milk would be seropositive for *Brucella* in Erbil Governorate if screened by MRT assay (Table 1). On contrary, ELISA assay found 9.7% of samples to contain anti-*Brucella* antibodies (Table 1).

Similarly, insignificant increase in frequency among goats was also noted in ELISA assay. According to indirect ELISA, 6.68% - 13.47% (95% CI) of sheep and goats in Erbil are expected to be seropositive for brucellosis by ELISA. There are no significant differences neither between the two serotests (p= 0.436) in terms of brucellosis detection, nor between the populations screened (Table 1).

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

Milk type	No. Examined	Positive samples No (%)	95% CI	*P* value*
MRT				
Sheep milk	150	16 (10.7)	6.22 - 16.74	0.054
Goat milk	170	21 (12.4)	7.81 - 18.26	
Total	320	37 (11.6)	8.27 - 15.58	
ELISA				
Sheep milk	150	13 (8.7)	4.70 - 14.36	0.054
Goat milk	170	18 (10.6)	6.40 - 16.22	
Total	320	31 (9.7)	6.68 - 13.47	

* *P* value less than 0.05 is considered significant.

Prevalence of *Brucella* species

The results indicated that the overall isolation rate of *Brucella* species from raw milk samples of sheep and goats was 7.8%. It is distinctly clear that finding rate in goat milk was higher than sheep milk samples. Successful bacteriological isolation of *Brucella* spp. indicates active brucellosis. The identified species of *Brucella* were *Br. melitensis* and *Br. abortus* without any significant difference in distribution in sheep or goats' milk samples (Table 2).

Diagnostic performance of MRT and ELISA

The Milk Ring Test technique(MRT) detected more cases of brucellosis 11.6% than the classical culture procedure 7.8% in both sheep and goat milk. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of MRT are given in table 3. The accuracy (efficiency) of MRT in detecting ovine and caprine brucellosis is 94.1% in comparison to the culture method. In addition to its accuracy, the high specificity of MRT test candidates it to be a simple alternative screening/diagnostic method for ruling out *Brucella* infection rather than confirming the infection in suspected animal/herd.

Table 2: Isolation of *Brucella* species from sheep and goat raw milk

Milk type	*Br. abortus* No. (%)	*Br. melitensis* No. (%)	Total No. (%)	*P* value
Sheep milk	4 (36.4)	7 (63.6)	11 (7.3)	0.935
Goat milk	5 (35.7)	9 (64.3)	14 (8.2)	
Total	9 (36.0)	16 (64.0)	25 (7.8)	

Table 3: The performance of the serological tests for detection of brucellosis in sheep and goats

	MRT (95% CI)	ELISA (95% CI)
Sensitivity	73.33(44.90-92.21)	84.62(54.55-98.08)
Specificity	95.86(91.21-98.47)	97.89(93.95-99.56)
PPV	59.99(39.27-77.66)	77.21(51.92-91.40)
NPV	97.70(94.83-98.99)	98.69(95.46-99.63)
Accuracy	94.10(89.25-97.21)	96.85(92.74-98.99)

PPV: Positive Predictive Value, NPV: Negative Predictive Value.

Temporal Dispersal of seropositive samples

Differences in the *Brucella* antibodies prevalence among the sheep and goat raw milk samples during six months were also investigated (Figure 1). In spite of the noticeable increase in frequency during autumn (September to November), no relationship between study period and brucellosis distribution was found statistically. However, autumn progress was related with declining in brucellosis ($r^2 = 0.867$).

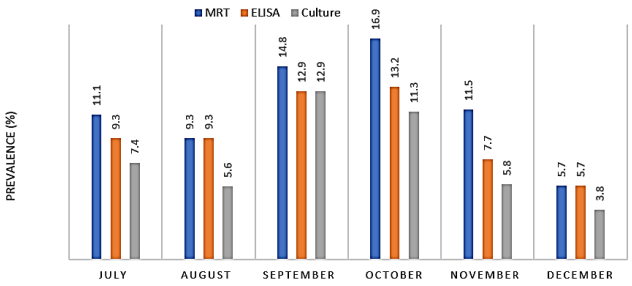

Figure 1: Distribution of raw milk positive samples for brucellosis during study period.

Discussion

Clinical human and animal brucellosis takes overabundance of synonyms like Malta fever, undulant fever, Mediterranean fever, Rock of Gibraltar fever, Neapolitan fever, Crimean fever, Corps disease, contagious abortion, and Bang's disease, and most of these names are still used in different regions of the world. It is one of the most significant zoonotic foodborne diseases in the world, brucellosis, is still present in Iraq, and is causing much distress to the country (2,10,13). This study addresses fast and reliable techniques for screening of *Brucella* antibodies among sheep and goats raw milk in Erbil Governorate.

On one hand, the general occurrence of *Brucella* antibodies in the raw milk samples was 11.6% and 9.7% according to MRT and ELISA, respectively. These findings are harmonious with published similar studies from Pakistan (14), Iraq (10,13,15), and India (16). On the other hand, higher rates of anti-*Brucella* antibodies were documented in studies in Libya 21% (17), India 27% (18), Egypt 36.4%-56.2% (19), and Iran 45% (20). Such variations might be attributed to various factors including the extent of encounters with wildlife (natural reservoirs), seasonality, level of available veterinary care, and testing methodology (10,13).

The incidence of *Brucella* among raw milk of sheep and goat at Erbil seems comparable to different populations. The overall isolation percentage of *Brucella* spp. in this work 7.1% is comparable to previous studies in Erbil ewes and nanny goats populations (10), and also in cow and buffalo populations (2). A Nigerian study also detected a similar prevalence in livestock by the bacteriological approach (21). Besides, different rates of *Brucella* contaminating sheep and goats' raw milk were found in Iran ranged from 1.2% to 45.5% (20,22). In addition to seasonal and epidemiological variations in different regions, isolation of *Brucella* is also influenced by the bacteriological protocol. Indeed, isolation from the milk samples may be enhanced if more than one of the culture medium is used (13). The observation that *Br. melitensis* is the predominant species is consistent with previous literature (10,13). Indeed, this observation may explain the difference in prevalence between goats and sheep populations in the present work.

MRT is an inexpensive serological test that was found to be 95.9% specific to rule out infection (91.2 - 98.5%, 95% CI). This finding is in good agreement with previous studies that found the sensitivity and specificity of MRT to be 85% and 95% in sheep and goat populations, respectively (12). However, an Egyptian large study found a complete sensitivity 100% when testing milk samples of cow and buffalo, whereas it dropped to 93% when milk samples from goats and sheep were tested (19). It should be noted that both of serological tests and culture method have a lower sensitivity in comparison to recent molecular diagnostic procedures, but this drawback is recompensed by the fact that the MRT is cheap and easy to perform (19). Meanwhile, ELISA and PCR approaches are expensive and unusual in several developing countries (23,24).

The seasonality of brucellosis is expected to be linked to human activities and climatic variability. Indeed, short sunshine duration and lower temperatures during winter and spring were found to be strongly related to temporal peaks in

the occurrence of brucellosis. The seasonality of brucellosis in sheep and goats was also reported to be attributed to the seasonal birthing (23). Moreover, human cases were found to be directly related to the parturition of small ruminants**.** On the contrary, other studies from northern hemisphere countries had reported an increase in the incidence of brucellosis during summer and spring (25).

According to (25), the mass vaccination is vital for the control and eradication of ovine and caprine brucellosis, also there are other complementary measures that may need attention include restriction and control of trade, improved farm hygiene, limit the movement of animals, testing of animals and isolation the infected animals. We must educate the farmers that the cornerstone of zoonotic infectious disease epidemiology is the One Health conception. One Health is not new, but it has become more important in recent years. It is a collaborative, multisectoral, and transdisciplinary program, working at the global national, and local levels, with the goal of attaining optimal health outcomes diagnosing the correlation between individuals, animals, plants, and their mutual environment. The eradication or control of the brucellosis among farming and wildlife animals is essential for the control of the disease in human populations.

Conclusions

Brucellosis still one of the most predominant zoonotic diseases that can seriously affect humans' health and food producing animals. The occurrence of brucellosis in ovine and caprine flocks was moderate in Erbil governorate. This percentage in sheep and goat milk is consider risky for public health. Consumers are highly recommended to properly heat raw milk before consumption in order to destroy this milk borne pathogen. Simple screening by MRT can be used for fast routine monitoring of lactating sheep and goats. The epidemiology and seasonal differences in brucellosis among raw milk of sheep and goats in Erbil is not completely clear. A whole-year wide study is recommended to provide more epidemiological data regarding the incidence, seasonality, and other associated factors. Advancement of health attentiveness through the media (audio, visual media, and newspapers) is advised to highlight the procedure of transmission and prevention of animal-to-human infections.

Conflict of interest

The authors have no conflict of interest.

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تشخيص داء البروسيلات في الحليب الخام للأغنام والماعز باستخدام تقنيات سريعة وموثوقة

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الخلاصة

أجريت هذه الدراسة لمعرفة مدى انتشار داء البروسيلات بين الحليب الخام للأغنام والماعز في محافظة أربيل من خلال الكشف عن الأجسام المضادة للبروسيلا وأنواع البروسيلا في عينات الحليب الخام من الأغنام والماعز. تم جمع ٣٢٠ عينة من الحليب الخام (١٥٠ من الأغنام و ١٧٠ من الماعز) بشكل عشوائي من الإناث المرضعات. كان الانتشار الإجمالي للأجسام المضادة للبروسيلا في عينات الحليب الخام للأغنام والماعز ١١,٦ و ٩,٧٪ وفقا إلى اختبار حلقة الحليب واختبار الاليزا غير المباشر على التوالي. بلغت نسبة العزل الكلية لأنواع البروسيلا من عينات الحليب الخام للأغنام والماعز ٧,٨٪. يشير العزل البكتريولوجي لأنواع البروسيلا إلى أن الأنواع التي تم عزلها هي *B. melitensis* و *B. abortus* دون أي فرق معنوي في التوزيع في عينات حليب الأغنام أو الماعز. وكشفت الدراسة الحالية عن وجود زيادة ملحوظة في معدل الانتشار خلال الخريف (أيلول إلى نوفمبر). لا يزال داء البروسيلا يمثل تهديدا مهما للصحة العامة. وبناء على أداء الفحوصات المستخدمة، توصي الدراسة بأهمية الاعتماد على اختبار حلقة الحليب في المراقبة الروتينية لداء البروسيلات في مصانع الألبان، حقول الإنتاج، والمراكز الخاصة بجمع الألبان. كما ينصح المستهلكون أيضا بضرورة بسترة الحليب الخام للقضاء على البروسيلا قبل الاستهلاك وكذلك قبل إجراء المعاملات التصنيعية على الحليب الخام.