The significance of milk ring test for identifying brucella antibodies in cows and buffaloes' raw milk at Erbil governorate, Kurdistan region, Iraq

D.A. Al-Mmashhadany

Department of Pathological Analysis, College of Science, Knowledge University, Erbil, Iraq

Abstract

This study was undertaken to monitor *Brucella* antibodies in the milk of cows and buffaloes in Erbil Governorate, Kurdistan Region, Iraq, using mik ring test (MRT) assay. A total of 210 samples of milk (130 from cows and 80 from buffaloes) were randomly collected from lactating females. The overall prevalence of *Brucella* antibodies in all the milk samples was 8.6% (18/210). The highest rate was 9.2% found in the cow milk (12/130), while the lowest rate was 7.5% of the buffalo's milk (6/80). Out of 210 milk samples, only 15 (7.1٪) were culture-positive for *Brucella*; about ٧.7% (10/130) and 6.3% (5/80) from cows and buffaloes respectively. In terms of comparison between MRT and standard milk culture method, MRT was found more sensitive (83%), specific (98%), with the accuracy of 97% in comparison to the employed culture approach to detect Brucellosis agents in milk. The results also revealed that 70% and 60% of isolates were *Brucella abortus*, while 30% and 40% were *Brucella melitensis* from the milk of cow and buffaloes respectively. The highest rate of frequency for *Brucella* antibodies according to MRT was found in February (12.1%), while the lowest rate was found in June (5.7%). This study emphasizes that Brucellosis is still a significant public health hazard in the Kurdistan region. The study recommends MRT adoption in routine monitoring of brucellosis in milk collection centre, dairy factories, and farm. Consumers are also recommended to sufficiently heat the milk to destroy this foodborne pathogen before consumption or industrial processing.

أهمية إختبار حلقة الحليب لتحديد الأجسام المضادة للبروسيلا في حليب الأبقار والجاموس الخام في محافظة أربيل، إقليم كردستان، العراق

ضاري عليوي المشهداني

قسم التحليلات المرضية، كلية العلوم، جامعة نولج، أربيل، العراق

الخلاصة

صممت هذه الدراسة لتحديد مدى إنتشار الأجسام المضادة للبروسيلا في حليب الأبقار والجاموس الخام في محافظة اربيل، حيث تم جمع ٢١٠ عينة حليب عشوائية من الاناث الحلوبة المتواجدة في القرى المحيطة بمدينة اربيل خلال الفترة ما بين يناير حتى يونيو ٢٠١٨، وشملت هذه العينات ١٣٠ عينة من الأبقار و ٨٠ عينة من الجاموس. وقد تم إجراء إختبار حلقة الحليب MRT، وكذلك عزل وتوصيف بكتريا البروسيلا على الأوساط الزرعية المناسبة. أظهرت النتائج ان إجمالي نسبة إنتشار الأجسام المضادة للبروسيلا في الحليب الخام إعتمادا على إختبار حلقة الحليب هي ٨,٦% (٢١٠/١٨)، وان نسبة إنتشار الأجسام المضادة للجرثومة في عينات الحليب الخام لكل من الأبقار والجاموس بلغت ٩,٢% (١٣٠/١٢) و ٧,٥% (٨٠/٦) على التوالي. في حين بلغت نسبة العزل الكلية لبكتريا البروسيلا ٧,١% (٢١٠/١٥) من عينات الحليب، شكلت منها ٧,٧% (١٣٠/١٠) من حليب الابقار و ٦,٣% (٨٠/٥) من حليب الجاموس. كما اشارت النتائج الى ان نسبة عزل *Brucella abortus* من حليب الابقار والجاموس كانت ٧٠% و ٦٠%، في حين كانت نسبة عزل *Brucella melitensis* ٣٠% و ٤٠% على التوالي. اما النتائج التي تخص العلاقة مابين نسبة إنتشار الاجسام المضادة للجرثومة في الحليب ومابين اشهر السنة التي تضمنتها الدراسة، فقد وجد ان أعلى نسبة إنتشار كانت خلال شهر فبراير وبنسبة ١٢,١% واقل نسبة كانت في شهر

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يونيو ٥,٧%. كما اوضحت نتائج الدراسة بأن إختبار الحلقة يمتلك حساسية ٨٣% ونوعية ٩٨% ونتائج دقيقة بنسبة ٩٧% بالمقارنة مع طريقة عزل البروسيلا من الحليب. ولقد بينت هذه الدراسة أن داء البروسيلات لا يزال يشكل أحد المخاطر الصحية التي تهدد الصحة العامة في إقليم كردستان. نوصي باهمية إستخدام اختبار حلقة الحليب لمراقبة البروسيلا سواءً في مراكز تجميع الحليب او في مصانع الالبان او في الحقول، كما تؤكد الدراسة على ضرورة بسترة الحليب قبل تناوله او تصنيعه بهدف القضاء على هذه البكتريا.

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Introduction

Brucellosis is a cosmopolitan bacterial zoonotic disease (caused by *Brucella* spp.) that affects humans and various species of the wild and domestic animals, particularly food-producing animals, including large and small ruminants such as cattle, buffaloes, camels, sheep, goats, pigs, and reindeer. Through the previous two decades, the infection has also been recognized in marine mammals, including beaked whales, dolphins, cetaceans, porpoises, and seals, which may present an emerging risk to individuals professionally exposed to contaminated tissues from such animals. This disease is highly infectious with a contagious dose of 10–100 cells are adequate to cause systemic infection [1,2].

Brucellosis is a foodborne and professional zoonotic disease, caused by the bacterial genus *Brucella*. This infection has an extremely emerging and significant reemerging potentials in numerous countries. In addition, it is the major cause of direct financial losses due to the major hindrance for international trade of milk, meat, and their products [3,4]. The transmission occurs through ingestion of contaminated milk or meat and from mothers to breastfed babies. The transmission of *Brucella* also occurs through mucous membranes or skin wounds, following direct contact with urine, vaginal discharges, blood, tissues, placenta, aborted fetuses, and through inhalation of airborne agents in an atmosphere [5,6]. Human brucellosis is a severely debilitating and disabling life-threatening disease. It is recognized by the clinical problems such as, the contribution of the interior organs, peripheral arthritis, bronchopneumonia, epididymitis, orchitis, hepatic abscesses, sacroiliitis, osteomyelitis, spondylitis, meningitis, encephalitis, cardiovascular complications, and prostatitis [7,8].

Brucellosis is found worldwide, but predominates in the countries of the Middle East, Mediterranean countries, Africa, Asia, Central and South Americas, however, some developed countries are essentially free of brucellosis [9,10]. The international map of human brucellosis has considerably changed over the last decade as a consequence of complex factors [11]. According to statistics of the World Health Organization (WHO), more than 500,000 new cases of brucellosis are registered worldwide annually. Nonetheless, several researchers estimate that the number of human brucellosis cases may be up to 26 times higher than the figure stated above [12].

Microbiologically, *Brucella* spp. are intracellular, non-motile non-sporing gram-negative short rods. They are aerobic, but some strains require 5-10% CO_2 for primary isolation. Growth *in vitro* is slow and primary isolation may require up to 4 weeks of incubation at 37°C. The colonies appear shiny surface on transparent media usually after 4-5 days of incubation. *Brucella* colonies are smooth, transparent, raised, convex with an entire edge, punctuate, and non-hemolytic. Biochemically, carbohydrates are not fermented and aerobic oxidation is the sole energy-producing process [13,14]. To date, twelve *Brucella* species have been reported each species may infect a different host group, but species have a preference to certain host category [15,16].

Recently, Jaff reported that the occurrence of human brucellosis in Kurdistan region, Iraq is still higher than recorded from bordering countries. *Brucella* infections have been reported from all three Iraqi Kurdistan governorates [17]. The same study had also pointed out that the frequency proportion of brucellosis among cattle in 2012 was 10.7% in Erbil city, 6.36% in Dohuk in 2011, and 976 cases among Sulaimani governorate in 2013. In Kurdistan region, people consume milk of the various animals including; cows, buffaloes, ewes, nanny goats, and camels which had been reported as a source of infection.

Milk Ring Test (MRT) was first qualified in Germany by Fleischhauerat 1937, it is the first-line routine checking test for individual dairy female and potentially infected flocks for brucellosis. MRT is an easy, simple, satisfactory, inexpensive, time-saving, and effective method to monitor brucellosis in milk-producing herds. It primarily detects IgA and IgM antibodies against *Brucella* spp. in raw milk. The sensitivity and specificity of MRT have reported to be 85% and 95%, respectively [18,19].

The study aimed to monitor the sero-prevalence of brucellosis among cattle and buffalo population in Erbil city and to calculate the sensitivity and specificity of MRT in comparison to traditional bacterial culture approach. The correlation between months of the study and frequency of *Brucella* antibodies in milk was also investigated.

Materials and methods

Samples Collection and Transport

A total number of raw milk samples was 210 (130 from cows and 80 from buffaloes) that were collected from lactating females from villages around Erbil city, during the

period from January to June 2018. The milk samples (100 ml for each) were collected into sterile plastic containers with screw lids under sterile hygienic conditions (20). All the samples were transported on ice to the Laboratory of Microbiology at Pathological Analysis Department, College of Science, Knowledge University.

Detection of *Brucella* antibodies

Detection of *Brucella* antibodies in raw milk was done by using MRT. The test was carried out by adding one drop (~ 0.05 ml) of MRT antigen (JOVAC Jordan) to 1 ml of whole milk in a narrow test tube 11 x 100 mm. The antigen milk mixtures were incubated at 37°C for 1-3 hours. If the specific antibody is present in the milk it binds 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 (20).

Isolation and Identification of *Brucella*

Isolation of *Brucella* species from the raw milk samples was done under sterilized conditions, following standard procedures (21). Inoculated 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 employed for identification purposes of the suspected isolates (22).

Sensitivity and Specificity of MRT

The sensitivity and specificity of the MRT were calculated according to standard equations, using the bacterial isolation diagnostic method as a gold standard (23).

Statistical analysis

Data were analyzed using SPSS software version 15, confidence intervals were estimated using normal distribution approximation at an alpha level of 0.05.

Results

Occurrence of *Brucella* antibodies

According to MRT, the overall rate of *Brucella* antibodies in raw milk samples was 8.6%. The percentage of positive samples among cows group was 9.2% which is higher than the percent found in buffaloes group. Statistically, it is estimated that 5% - 12% (95% confidence interval) of the cows and buffaloes would be seropositive for *Brucella* in Erbil governorate if screened by MRT assay (Table 1).

Table 1: Occurrence of *Brucella* antibodies among cow and buffaloes raw milk according to MRT

Type of Milk	No	Positive (%)	Negative (%)
Cow	130	12 (9.2)	118 (90.8)
Buffalo	80	6 (7.5)	74 (92.5)
Total	210	18 (8.6)	192 (91.4)

Occurrence of *Brucella* spp. in the milk samples

The overall isolation proportion of *Brucella* species from raw milk samples was 7.1% (15/210). It is obviously clear that detection rate in both groups (cows and buffaloes)is more similar to each other (difference between groups is less than 1%). Regarding the identified species of *Brucella* from raw milk samples, *Br. abortus* comprised two thirds 66.7% of total isolates (10/15 isolates), while the remaining isolates were of *Br. melitensis* (Table 2).

Comparison of MRT to culture approach

The MRT assay detected more cases of brucellosis 8.6% than traditional culture method 7.1% in both groups of cows and buffaloes. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of MRT are given in Table 3. The efficiency (accuracy) of MRT in detecting bovine brucellosis is 97% in comparison to culture method, which candidates the MRT to be a good alternative screening/diagnostic method.

Temporal Distribution of seropositive samples

Variations of *Brucella* antibodies occurrence in raw milk samples during six months have been investigated (Table 4). The highest rate of incidence of *Brucella* antibodies detected by MRT was found in February 12.1%, while the lowest rate was documented in June 5.7%. Accordingly to the statistical calculations, there is a good correlation ($r^2 = 0.87$) between the months and prevalence of brucellosis.

Table 2: Isolation of *Brucella* species from cow and buffaloes raw milk

Type of Milk	No	Positive (%)	Negative (%)	*Br. abortus* n (%)	*Br. melitensis* n (%)
Cow	130	10 (7.7)	120 (92.3)	7 (70)	3 (30)
Buffalo	80	5 (6.3)	75 (93.7)	3 (60)	2 (40)
Total	210	15 (7.1)	195 (92.9)	10 (66.7)	5 (33.3)

Table 3: The relationship between result of MRT and isolation of *Brucella* species from cow and buffalo's milk

Type of Milk	No.	MRT positive n (%)	Culture positive n (%)	Sens.	Spe.	PVP	PVN	Effic.
Cow	130	12 (9.2)	10 (7.7)	83.3%	98.3%			
Buffalo	80	6 (7.5)	5 (6.3)	83.3%	98.7%	83%	98.5%	97%
Total	210	18 (8.6)	15 (7.1)	83.3%	98.5%			

Sens; Sensitivity, Spe: Specificity, PVP; Predictive value positive, PVN; Predictive value negative, Effic.; Efficiency.

Table 4: Relation between months and prevalence of *Brucella* antibodies according to MRT during period of study

Month	No. Examined Cow positive (n)	No. Examined Buffaloes positive (n)	Total examined	Total positive n (%)
January	22 (3)	13 (1)	35	4 (11.1)
February	20 (2)	13 (2)	33	4 (12.1)
March	22 (2)	14 (1)	36	3 (8.3)
April	23 (2)	14 (1)	37	3(8.1)
May	21 (2)	13 (0)	34	2 (6.1)
June	22(1)	13 (1)	35	2 (5.7)
Total	130 (12)	80 (6)	210	18 (8.6)

Discussion

Brucellosis is a zoonotic disease affected domestic and wild animals. It is mainly a disease of food producing animals such as cattle, buffalo, camels, sheep, goats, and swine. Transmission to humans occurs in various ways, mostly via ingestion of contaminated food such as raw milk or meat or their products (9).

The difference in percentage of *Brucella* antibodies between cows 9.2% and buffaloes 7.5% groups is not significant and mostly owing to difference in sample size between cows and buffaloes groups. The result of overall occurrence is in agreement with previous studies from Yemen (20), Pakistan (24), and Kenya (25) where prevalence of *Brucella* antibodies ranged from 7% to 9.7% detected by MRT assay for raw milk samples from cattle and buffaloes. Indeed, lower rates of prevalence of *Brucella* antibodies were also reported from different countries. For instance, a Zimbabwean study (26) documented a 1.7% from a large sample size. While other reported rates ranged from 3% to 6% from Pakistan (27,28) and India (29,30). On the other hand, slightly higher incidences were reported in India 10.5% (31), Nigeria 15%, Yemen 16%, and India 18% (32-34). Moreover, higher prevalence rates were reported in India 27% (35), Uganda 33.5% (36), and in Egypt 51% and 49.8% for cows and buffaloes screened by MRT with an overall incidence of 47.8% (23). Such differences in prevalence may be attributed to many factors including husbandry and rearing practices, adherence for vaccination programs, herd size, cattle age and parity, among others (37,38).

Isolation of *Brucella* is a difficult, tedious, time-consuming, and potentially risky laboratory work. Therefore, most recent studies employ culture-independent diagnostic assays such as ELISA and PCR to detect the infection. The overall isolation rate of *Brucella* spp. in this study 7.1% is similar to a Nigerian study that detected brucellosis in livestock by the bacteriological approach (39).

It is well-known that *Br. abortus* has a preference for cattle over other ruminants, while *Br. melitensis* is the usual causative agent of brucellosis in goat and sheep animals (13). However, when cattle and buffalo are reared and co-housed with herds of goats and sheep, *Br. melitensis* infect and establish the disease in the cattle much similar to *Br. abortus* (40,41). Currently, a growing literature reporting the isolation of *Br. melitensis* from cattle is emerging worldwide but at low rates (42-46). Indeed, an Iraqi recent study isolated both of *Br.abortus* and *Br. melitensis* (overall rate is 3%) from milk products (47). The isolation rate of *Brucella* in the present study is two-fold higher 7.1% than the previously mentioned Iraqi study. This difference may be attributed to the fact that *Brucella* cells may have been killed during industrial treatments for production of sampled milk products in the previous study. Additionally, sampling different area with different *Brucella* epidemiology or during the dry season may account for such variations in isolation rates. The isolation of *Brucella* from milk samples may be improved if more than one culture medium is used. On the other hand, higher isolation rates were also reported from different countries. In Syria, a recent study isolated *Br. melitensis* from bovine raw milk samples at a rate of 25% (48). Furthermore, in San Paulo,

30% of bovine screened milk samples yielded *Br. abortus* during a study of four years (49).

The sensitivity 83.3% and specificity 98.5% of MRT in comparison to culture approach clearly reveal its good value as a straightforward, inexpensive screening test to detect brucellosis in raw milk of cattle and buffaloes. However, a higher sensitivity 100% and lower specificity 75-73.5% has been reported for the MRT testing of cow and buffalo milk samples (23). It should be noted that both of culture method and MRT have low sensitivity in comparison to current molecular diagnostic techniques, but this drawback 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. However, a recent Syrian study has found that PCR and culture approach yielded the same results while the MRT showed lower rates of positive results (48).

The temporal distribution of seropositive raw milk samples from cows and buffaloes shows good correlation (r^2 = 0.87) between the months and prevalence of brucellosis. The gradual decrease in the sero-positive rates of brucellosis in raw milk could be attributed to the gradual increase in temperature and/or gradual decrease in humidity and rain level in Kurdistan region during summer-autumn elapsing. For instance, 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 (50). Larger sample size of milk samples for a complete year may reveal a clearer picture in term of *Brucella* prevalence rates and any association with certain months if any. To the best of author's knowledge, no study has monitored the bovine prevalence of brucellosis in a time span in Iraq or nearby countries. Moreover, There is scarcity of published data on the changing sero-prevalence of bovine brucellosis among seasons. Consequently, comparing and contrasting the finding of time-related seropositive rates is not possible currently.

In conclusion, the rate of brucellosis in cattle and buffalo population in Erbil Governorate is high and hazardous to humans. MRT can be used for rapid everyday monitoring of lactating cows and buffaloes due to its simple procedure for routine screening of Brucellosis in raw milk, and it its efficiency in comparison to laborious culture-based diagnosis especially in collection centers of milk and in dairy factories. Based on the presented findings, sufficient heating of raw milk is highly recommended to destroy this milk-borne bacterium. The epidemiology and seasonal variations in brucellosis rates in cattle and buffalo in Erbil is not fully clear. Further studies addressing this issue are highly recommended. Promotion of health awareness through the media (visual media, audio, and newspapers) is ad advised to highlight the method of transmission and prevention of animal and human brucellosis in order to control the disease in the food producing animals.

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