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THE ROLE OF MILK RING TEST IN MONITORING BRUCELLOSIS AMONG COW MILK IN ERBIL GOVERNORATE / KURDISTAN REGION/IRAQ

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ABSTRACT

This work was designed to screen Brucellosis among cow milk in Erbil by using of MRT. A total of 220 raw milk samples were collected during April 2017 to September 2017, these included 140 samples from randomly selected lactating cows at small villages around Erbil city and 80 samples from cow milk sold at different retail markets in Erbil city. The overall prevalence of Brucella antibodies in cow milk samples was (7.3%). Brucella antibodies were (7.9%)and(6.3 %) in milk from villages and retail markets consecutively.Out of 220 cow milk samples, only (5.9 %) Brucella isolates were found, this includes (7.1%) positive samples from villages and (3.8 %) positive samples from retail markets.

The relation between result of MRT and isolation of Brucella species indicated that (7.9 %) samples from villages were positive according to MRT compared with (7.1 %) samples gave isolates of Brucella species , and (6.3 %) from retail markets were positive according to MRT compared with (3.8 %) samples gave isolates . Also, our result confirmed that (80%) and (66.7 %) were Brucella abortus, while (20%) and (33.3 %) were Brucella melitensis isolated from villages and retail markets consecutively.

The highest rate of prevalence of Brucella antibodies was found in July and August (12.5%), while the lowest rate was found on May (3.3 %).We concluded that MRT plays an important role in the detection of Brucellosis in milk.

Keywords:- Milk Ring Test, Monitoring, Brucellosis, Cow Milk, Erbil city, Kurdistan Region, Iraq

1-INTRODUCTION

Brucellosis is a cosmopolitan bacterial zoonotic disease that affects humans and various species of Wild and domestic animals especially food-producing animals including Cattle, Sheep, Goats, Camels, Buffaloes, Pigs and Reindeer. Brucellosis is a foodborne and occupational zoonosis, so it recognizes a public health problem and one of the major causes of high morbidity and mortality. It is also a major cause of direct economic losses resulting from clinical disease, abortion, neonatal losses, reduced fertility, decreased milk production. Brucella infection is responsible for up to 20 – 25 % decrease in milk production, 10 – 15 % in meat production, it is also a major impediment for international trade of milk, meat, and their products [1- 3].

Brucellosis is a highly emerging infectious disease (EID) and one of the most important reemerging zoonoses in many countries , and the global map of human brucellosis has drastically changed over the past decade , because of a complex multifactorial set of changing circumstances such as lack of various sanitary conditions , the standard of socio-economic activities , and political reasons , together with increased globalization , with persons, animals, and their products moving around the world [4-6].

It is an important human disease found around the world, particularly Mediterranean basin countries, the Middle East including Iraq and Iraqi Kurdistan, Arabian Gulf, Africa, Asia, Central and South Americas, and yet it is often unrecognized and frequently goes unreported. There are a few countries in the world that are officially free of the disease such as Australia, Canada , Japan , Cyprus , Denmark , Finland , The Netherlands , New Zealand , Norway , Sweden and the United Kingdom , although cases still occur in people of these countries returning from endemic region [7- 9].

A high prevalence of Brucellosis in certain geographic areas is well recognized, although largely underestimated. According to World

Health Organization (WHO) data more than 500, 000 new cases of this disease are registered in the world every year. Many researchers reported that this figure underestimates the magnitude of the problem, and some of them estimated that the number of human Brucellosis cases may be up to 26 times higher than the above number of cases [10 – 13].

The disease is caused by different species of the genus Brucella, which tend to be host-specific. Brucellae are gram-negative coccobacilli or short rods with straight or slightly convex in shape and rounded ends, facultative intracellular, nonspore forming, non - motile, urease +ive, aerobic but may need added CO_2, and encapsulated [14–16].

Eleven species are currently described in the genus Brucella, each one may infect different host species, but each Brucella species has a preference for its host species. Six classical out of eleven species include Br.abortus, Br. melitensis, Br. suis, Br. neotomae, Br. ovis, Br. canis and five novel species of Brucella include Br. ceti, Br. pinnipedialis, Br. microti, Br. inopinata, Br. papionis [17-19].

Recently, [20] mentioned that the prevalence of human Brucellosis in Iraqi Kurdistan is still higher than records from neighboring countries, and it has been reported from all three Iraqi Kurdistan provinces. He mentioned that the prevalence rate in Erbil city was 10.7% in 2012, in Dohuk was 6.36% in 2011, and 976 cases were recorded in Sulaimani province in 2013.

2. AIMS AND OBJECTIVES:

The consumption of contaminated milk and dairy products has been widely documented as an important route of Brucella transmission. In particular, unpasteurized milk or milk products from infected cows have been considered a source of infection for the general population, especially in developing countries, therefore the goals of this research were to study the prevalence of Brucella antibodies and Brucella species among cow milk in Erbil Governorate, and to determine the relationship between prevalence of Brucella antibodies in milk with months during the period of study. Also high lights on the hazard of Brucella help in understanding the role of milk and dairy products in the transmission of Brucella to human, and to focusing on the importance of milk pasteurization.

3-MATERIALS AND METHODS

3.1. Study Design and Sampling

A total of 220 raw milk samples were collected under sterile hygienic conditions

during April 2017 to September 2017, these included 140 samples from randomly selected lactating cows at small villages around Erbil city and 80 samples from cow milk sold at different retail markets in Erbil city. The samples were collected under sterile hygienic conditions according to [21]. Each sample was collected into sterile 10 ml plastic tubes with screw cups. The samples were transported to the pathological Analysis Department, College of Science, Knowledge University, Erbil City.

3.2. Detection of Brucella antibodies in milk

In the laboratory, the detection of Brucella antibodies in milk was done by using Milk Ring Test (MRT). The test was carried out according to [1]. One drop (0.03 ml) of hematoxylin - stained antigen is mixed with 1 mL of milk in a narrow test tube (11 x 100 mm). Incubate at 37˚C for 1 – 3 hours. 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 column of milk (Ring Blue and column white = Positive result; Ring white and column Blue = Negative result).

3.3. Isolation and Identification of Brucella

The isolation of *Brucella* from milk was done under sterile conditions according to [22, 23]. The identification of *Br. abortus and Br. melitensis* were confirmed by Biochemical analysis, and the tests performed illustrated in Table (A).

3.4. Statistical analysis

Data were analyzed using Chi-Square test and SPSS software version 15.

Table (A):- Phenotypic characteristics of *Brucella* species isolated from Cow milk

Biochemical tests	Br.abortus	Br.melitensis
Catalase test	+	+
Oxidase test	+	+
Indole test	-	-
Simmon's citrate	-	-
Urease activity	+ive, hydrolyzing urea within 1- 2 hours	Variable
Growth on MacConkey agar	-	-
Blood hemolysis	-	-
H2S production	+	-
Nitrate reduction	+	+
Triple Sugar Iron	-	-
Agglutination with monospecific sera A	+	-
Agglutination with monospecific sera M	-	+
Thionin	+	-
CO2 requirement	+	-

4- RESULTS

The overall prevalence of Brucella antibodies in cow milk samples was 16 / 220 (7.3%).The highest rate of prevalence of Brucella antibodies was found in milk samples from villages 11 (7.9%), while the lowest rate of prevalence was from the retail markets 5 (6.3 %) (Table 1).

From Table 2, we showed that among 220 samples of cow milk, only 13 (5.9 %) Brucella isolates were found. This result includes 10 (7.1 %) positive samples from villages and 3 (3.8 %) positive samples from retail markets.

On studying the relation between result of Milk Ring Test and isolation of Brucella Species from Cow Milk, it was found that 11 / 140 (7.9 %) and 5 / 80 (6.3 %) samples from villages and retail markets were positive according to MRT, compared with 10 (7.1 %) and 3 (3.8 %) samples gave isolates of Brucella Species respectively (Table 3).

Depending on Phenotypic characteristics of *Brucella* abortus and Brucella melitensis isolated from Cow milk , we achieved that 8 / 10 (80%) and 2 / 3 (66.7 %) of isolates were Brucella abortus, while 2 / 10 (20%) and 1 / 3 (33.3 %) were Brucella melitensis respectively (Table 4) .

Table 5 illustrate that the relationship between months and Prevalence of Brucella antibodies in milk samples during the period of study. From this table we noticed that the highest rate of prevalence of Brucella antibodies according to MRT was found in July and August 5 / 40 (12.5%), then in June 2 / 30 (6.7%), September 2 / 40 (5.0 %) , while the lowest rate was found in May and April 1/ 30 (3.3 %) and 1/40(2.5%) respectively .

Table (1):- Prevalence of Brucella antibodies Among Cow Milk According to MRT.

Collection Site	No. of samples Examined	+ive samples No.	%	-ive samples No.	%	Chi square	P value
Villages	140	11	7.9	129	92.1	99.46	0.00
Retail markets	80	5	6.3	75	93.7	61.25	0.00
Total	220	16	7.3	204	92.7	160.66	0.00

Table (2):- Isolation of Brucella species from cow milk in Erbil City.

Collection Si	No.exam	+ive culture No.	%	-ive culture No.	%	Chi square	P value
Villages	140	10	7.1	130	92.9	99.46	0.00
Retail marke	80	3	3.8	77	96.2	68.45	0.00
Total	220	13	5.9	207	94.1	167.56	0.00

Table (3): The Relation Between Result of MRT and Isolation of Brucella Species from Cow Milk

Collection Site	No.exam	Result of MRT No.	%	Isolation of Br SPP No.	%	Chi-Squar	P Value
Villages	140	11	7.9	10	7.1	64.00	0.00
Retail markets	80	5	6.3	3	3.8		
Total	220	16	7.3	13	5.9		

Table (4):- Prevalence of Brucella species in Cow Milk According to Collection Site

Collection Site	No.Isolated	Br. Abortus No.	%	Br. melitensis No.	%	Chi Square	P Value
Villages	10	8	80	2	20	2.27	0.32
Retail markets	3	2	66.7	1	33.3	0.33	0.56
Total	13	10	76.9	3	23.1	2.57	0.11

Table (5): Relationship between Months and Prevalence of Brucella antibodies (MRT) during the period of study

Month	Villages		Retail markets		Total examine Samples	Total +ive		Chi Square	P Valu
	No. exam	No. +ive	No. exam	No. +ive		No.	%		
April	25	1	15	0	40	1	2.5	36.10	0.00
May	20	1	10	0	30	1	3.3	26.13	0.00
June	20	2	10	0	30	2	6.7	22.53	0.00
July	25	3	15	2	40	5	12.5	22.50	0.00
August	25	3	15	2	40	5	12.5	22.50	0.00
September	25	1	15	1	40	2	5.0	32.40	0.00
Total	140	11	8	5	220	16	7.3	160.66	0.00

5- DISCUSSION

Brucellosis is primarily an animal disease and the transmission to humans occurs through different routes. Foodborne transmission is the most common way in which people become infected and results from the consumption of unpasteurized milk or milk products and raw or undercooked meat from infected animals. Transmission also occurs through skin wounds or mucous membranes, following direct contact with tissues, blood, urine, vaginal discharges, aborted fetuses or placenta, and through airborne infection in settings such as laboratories and slaughterhouses. Accidental inoculation of live vaccines, such as Br. abortus strain 19 and Br. melitensis Rev 1, can also occur, resulting in human infections. Transmission may also occur through venereal and congenital infection in humans. Infected mothers who are breastfeeding may transmit the infection to their infants, the transmission may also occur via tissue transplantation or blood transfusions, Person-to-person spread of brucellosis is extremely rare [24- 26].

Milk Ring Test (MRT) was first described by Fleischhauer in German in 1937, it is the best test for screening individual dairy cattle and potentially infected herds for Brucellosis. Milk Ring Test is a simple, easy, satisfactory, inexpensive, effective method, and takes low time to perform, and is usually the method of choice for the surveillance of dairy herds, it mainly detects IgM and IgA antibodies against *Brucella* infection in fresh milk. The MRT reported having a sensitivity of 85% and specificity of 95% [22, 27, 28].

In the work at hand, Two hundred and twenty 220 raw milk samples were collected from Erbil Governorate, Erbil, Kurdistan region, during the period from April 2017 to September 2017. These

included 140 samples from randomly selected milking cows at small villages around Erbil city and 80 samples from cow milk sold at different retail markets in Erbil city. The overall prevalence of Brucella antibodies in cow milk samples was 16 / 220 (7.3%). The highest rate of prevalence of Brucella antibodies was found in milk samples from villages 11 (7.9%), while the lowest rate of prevalence was from the retail markets 5 (6.3 %) (Table 1). The obtained results indicated that there was a significant difference at the level of 0.05 for the prevalence of Brucella antibodies in cow milk according to MRT, where the value of Chi-Square was (160.66) with the level of significance 0.000 ($p < 0.05$).

Our result was approach with percentage found by [29], who found that among 70 samples, only 5 milk samples (7.1%) were determined as positive by PCR. Also, [30] in Pakistan, reported that the prevalence of Brucella antibodies in cattle raw milk samples were 5 / 70 (7.1%). The result of our study was agreement with study in Kenya where the prevalence of *Brucella antibodies in cow raw milk* was 16 / 208 (7.7 %) (5), also our result was consistent with (21) in Yemen who found the prevalence of *Brucella antibodies in cow raw milk* was 25 / 300 (8.3 % %) .

In another hand, our results showed a less rate compared with the study conducted by [31] in Egypt who found that the prevalence of Brucella antibodies among raw cow milk was 55.8 %, and it was evident that all samples which were positive to culture and PCR assay were positive also to the MRT. [32] in India observed that the prevalence of *Brucella antibodies* in raw cow milk was 57 %. Also our result incompatible with the result achieved by [33]In Yemen, who reported that the prevalence of Brucella antibodies in cow milk was 10 / 63 (15.9 %). [34] in Nigeria, observed that the total prevalence of Brucella antibodies was 15.7% among cow milk samples, 17.7% of milk samples from herds and 12.5% samples from milk vendors.

[35] in India, mentioned that the overall prevalence of Brucella antibodies in cow milk was 18.61 %, and [36] in India noticed that 23 / 85 (27.05%) of the milk samples were positive by MRT. Another study in India [6] found that the total rate of Brucella antibodies in cow milk was 10.53 %. In Uganda [37] reported that 62/185 (33.5%) of raw cow milk were positive by MRT.

While the result achieved in our study was more than those reported in other countries, the prevalence of Brucella antibodies in raw cow milk according to MRT in India

was 17 / 500 (3.4 %) [3] , 12/302 (3.97%) in Pakistan [38], 21/ 483 (4.35 %) in India [39] .

However, lactating cows play an important role in the epidemiology of human Brucellosis, because the Brucella species localize in the supra mammary lymph nodes and mammary glands in more than 80% of infected cows, which continue to excrete these pathogens in their milk throughout their lives , so these bacteria can be transmitted to consumers via milk and dairy products which represents an important source of health hazard to community [40 – 42] .

At the same time, milk is a typical medium to test, because it is ready, inexpensive and directly obtained , also MRT can be done regularly several times, as well as this test gives a good reflection of blood serological tests, for this reasons MRT remains the most practical method to screen milking cows and confirm diagnosis of Brucellosis [22, 43].

From the result prevailed in Table 2, we show that Brucella isolates were found in 13 (5.9 %) among 220 samples. This result indicated that the isolation of Brucella species was high in cow milk samples from villages (7.1 %) compared with samples from retail markets (3.8 %). There is a significant difference at the level of 0.05 for the isolation of Brucella species from cow milk in Erbil City, where the value of Chi-Square was (167.56) with the level of significance 0.000($p< 0.05$).

In the study conducted by [44], they observed that 15 / 49 (30.61 %) of milk samples contained Brucella abortus. Also, our results (6.4 %) consider lesser than results shown by [45] who found that the Brucella DNA was detected in 10.3 % of 564 cow milk samples by real-time PCR. In another hand, the result achieved by [46] illustrated that the total percent of Brucella species in milk samples collected at winter season was 5 (2.5 %) which is regarded lesser than our result.

Anyway When lactating cows are infected with Brucella species, their milk is polluted with this type of bacteria , and Cows remain carriers and shed the Brucella in their milk for prolonged periods, besides if the milk is not pasteurized, these bacteria can be transmitted to people who drink milk or consume dairy products made from it [47- 48].

when we study the relation between result of Milk Ring Test and isolation of Brucella Species from Cow Milk , we found that 11 / 140 (7.9 %) and 5 / 80 (6.3 %) samples from villages and retail markets were positive according to MRT, compared with 10 (7.1 %) and 3 (3.8 %)

samples gave isolates of Brucella Species respectively (Table 3). There is a significant difference in Relation Between Result of MRT and Isolation of Brucella Species from Cow Milk ($p< 0.05$).

[49] in Syria partially consistent with our results as he indicated that from 2372 milk samples collected over a 6 – year period (2002–2007) from Syrian cow herds. The results were 57 %, 25 %, and 25 % for MRT, Culture, and PCR consecutively.

Also, [46] in Egypt reported that from 200 samples of milk were screened for Brucella antibodies as well as with culturing during summer and winter seasons (100 each). 12 samples were positive for MRT during the summer season in contrary no samples showed Brucella organisms growth after culturing on specific media. While In winter they reported that 17 samples Were positive for MRT, and 5 / 17 samples showed growth on specific Brucella medium.

According to the Table 4 , we noticed that 8 / 10 (80%) and 2 / 3 (66.7 %) of isolates were Brucella abortus , while 2 / 10 (20%) and 1 / 3 (33.3 %) were Brucella melitensis respectively . These observations indicate that Brucella abortus was the predominant species in cow milk. There is no significant difference between the prevalence of Brucella species (Br.abortus and Br. melitensis in cow milk according to collection site ($p> 0.05$).

Our result was inconsistent with research of [50], who revealed that Brucella melitensis biovar 3 is the only isolated species from Forty random samples of light salt white soft cheese and yoghourt. While in the study designed by [51] in Basrah, they isolate 9 *Brucella species* (3%) out of 300 milk product samples (8 from soft cheese and one from cream, No Brucella strain was isolated from ice-cream). The species and biotypes of these isolates were determined and it was found that 4 isolates of *Brucella abortus* biotype 4 and 5 isolates of *B. melitensis* biotype 2.

Another point of this study includes the relationship between months and Prevalence of Brucella antibodies among milk samples during the period of study in Erbil Governorate were followed up. Results which described in Table 5, explain that the prevalence increased in July and August 5 / 40 (12.5%). Then in June and September, prevalence rate were 2 / 30 (6.7%) and 2 / 40 (6.7 %) respectively . while the lowest rate were found in May and April 1/ 30 (3.3 %) and 1/40(2.5%) respectively . With ($p< 0.05$) there is a significant difference in the relationship between Months and prevalence of

Brucella antibodies during the period of study.

The results at hand consistent with the study conducted by [52] in Saudi Arabia, who found that the number of cases was highest in April to June ($n = 361$; 39.5%) and the lowest reported cases were in January. Also when [53] studies the trends of reported human cases of brucellosis, Kingdom of Saudi Arabia, 2004– 2012, noticed that most cases were reported during spring and summer seasons. In support of our finding, [54] in Kenya reported that the highest cases of brucellosis occurred during the month of July, followed by September, March, and October. They noticed that the most of the high incidences of brucellosis occurred during the rainy season, while the lowest cases were observed in December, followed by May and January.

[55] in Hamadan Province, Iran, noticed that new cases of human brucellosis increased to 93.7% in spring then 94.5% in summer, decreasing to 93.8% in autumn and 91.45% in winter; the highest relative frequency of new cases was observed in the summer season. In contrast, the relative frequency of recurrent cases was 6.21% in spring, 5.49% in summer, 6.18% in autumn, and 8.55% in winter. Also, [56] in China, from a total of 513,034 human brucellosis cases were recorded, of which 99.3% were reported in northern China during 1955–2014, and 69.1% (258, 462/374, 141) occurred during February–July in 1990–2014.

In Iran [57], found that from 176 patient cases, 94.8% of the people lived in rural areas and5.2% lived in the urban. Most reported cases were in June and July and the lowest statistic occurred in January. It seems that the disease process starts in the spring and in the summer reaches its peak and, then, begins to decline in autumn.

From this study, we concluded that MRT plays an important role in the detection of Brucellosis in milk. The overall prevalence of Brucella antibodies among cow milk in Erbil Governorate seems to be high (7.3%), and this percentage consider dangerous on public health.

Due to the importance of this study, we recommend that the consumers, particularly at rural districts, should remember that milk needs to be properly heated to destroy this foodborne pathogen, and dissemination of health awareness through the media (audio, visual media and newspapers), highlighting the mode of transmission of these bacteria. This study also emphasized on inter MRT in the diagnosis of this bacteria especially in collection centers of milk, in dairy factories, and in the field by

veterinarians to eradication and control of brucellosis in dairy cows.

6- CONCLUSION

From this study, we concluded that MRT plays an important role in the detection of Brucellosis in milk. The overall prevalence of Brucella antibodies among cow milk in Erbil Governorate seems to be high (7.3%), and this percentage consider dangerous on public health.

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