Molecular and serological diagnosis of *Brucella* among dairy Women Seller and their Families in Kut city

Khairi.J.W.AL-Ruaby

Department of Biology , Faculty of Science, University of Wasit

عدوى البروسيلا في النساء البائعات الألبان وعائلاتهم في مدينة الكوت

خيري جميل وحيد الربيعي

قسم علوم الحياة ، كلية العلوم ، جامعة واسط

المستخلص

عدوى البروسيلا (الحمى المالطية) هو المرض الذي يعتمد أساسا على الأفراد الذين يعملون وعلى اتصال مباشر مع الحيوانات. كان الهدف من هذه الدراسة للتحقيق في انتشار أنواع البروسيلا في النساء البائعات الألبان وعائلاتهم من خلال اختبار التفاعل البلمره المتسلسل الزمني PCR. أجريت الدراسة في مدينة الكوت (العراق)، حيث تم جمع عينات من مصل دم النساء اللواتي يبيعن منتجات الألبان (البائعات المتجولات) في أسواق المدينة والمقيميات في أربع قرى مختلفة حول المدينة، وكذلك أسرهم الذين يعيشون في اتصال مع الماشية والمشاركين في صنع منتجات الألبان. حيث تم جمع 186 (30 امرأة بائعات الألبان +156 فرد من أسرهم) وهي عينات من مصل الدم للفترة مابين تموز 2015 الى تشرين الاول لنفس العام ، باستخدام اختبار سيرولوجي وهو روز البنغال Rose Bengal Test (RBT).

وكانت النتائج 30 من النساء البائعات الألبان 6(٪20) مصابة بالبروسيلا ومن أصل 156 شخص (الأسر من النساء البائع الألبان) فكانت 44 (28٪). جميع العينات الموجبة لاختبار الروز بنغال اختبرت بتفاعل البلمره المتسلسل الزمني PCR. تم تزويد الجهاز بنوعين من الجينات لكشف عن *B. melitensis* . *B. abortus*.. جين BME 110466 لكشف عن *B. meltitensis* وجين Bru-Ab_0168 لكشف عن *B. abortus*. ومن مجموع من (50) عينة موجبة كانت *B. meltitensis* في 27(54%) عينة، و *B.abortus* في 11(22%)عينة. فيما (20)من النساء (6 بائعات +14 من أسرهم) مصاب بالبروسيلا أظهر الاستبيان 10 (50٪) منهم قد أجهضت و2 (10٪) ولدت جنين ميت. ان البروسيلا التي تصيب الإنسان تكون ذات الأهمية في التأثير على الصحة العامة في العراق والمزيد من الاهتمام ينبغي أن يركزاكثر على المرض. حيث تقدم الدراسة الحالية أساسا لدراسات أوسع لتقيل العدوى بالبروسيلا بين سكان الريف في العراق.

Abstract

Brucella infections (Brucellosis) is a disease that reaches individuals who work directly in contact with animals. The aims of this study was to investigate the prevalence of *Brucella* species in dairy women seller and their families by Real Time quantitative-qPCR reaction. The study was done in AL-Kut city (Iraq), Serum samples were collected from some women who sell dairy products (hawkers) in city markets and resident in different four villages around city, As in addition their families who live contact with cattle and participated in make the dairy products. One hundred eighty six (30 dairy women seller +156 of their families) Serum samples were collected between July 2015 to November 2015, and the Rose Bengal test (RBT) was performed on each sample.

A total of 30 dairy women seller (DWS) were 6 (20%) *Brucella* seropositive, out of 156 persons (families of dairy women seller) were 44 (28%) *Brucella* seropositive. All seropositive test by Real Time- PCR reaction, PCR was based on unique genetic loci of *B. melitensis and B. abortus*. The regions were chosen for the construction of primers and probes for species differentiation: BMEII0466 gene for *B. melitensis* and Bru-Ab2_0168 gene for *B. abortus*. Out of (50) Seropositive *B. meltitensis* was diagnosed in 27(54%) sample , *B. abortus* in 11(22%). sample . Among the (20) women (6 DWS+14 of their families) were seropositive. The questionnaire showed 10 (50%) of them had aborted and 2 (10%) presented with a stillbirth. Human brucellosis appears to be of public health importance in Iraq and more attention should be drawn on the disease. The current study provides a basis for larger studies to establish the incidence of *Brucella* infections among rural population in Iraq.

Key Words : Brucella, **dairy Women Seller**

Introduction

Brucellosis was zoonosis disease transmitted by contact with infected animals or consumption of contaminated milk or dairy products. Humans get infected by *Brucella abortus*, *Brucella melitensis, Brucella suis* and *Brucella canis*, however, *B. mellitensis* is the most common cause of infection in the

world , particularly in developing countries. (1). *B. aborutes* cause chronic disease in human , the bacteria enter by phagocytes and attach itself to the endoplasmic reticulum of these cells and cause the cell to resist self-death and these pathogens become resistant within cells of immune system (2). In humans, The clinical manifestations are varied and nonspecific. they caused an intermittent fever, joint pain (including arthritis), sweats, fatigue, malaise, anorexia, weight loss, headache, neurologic manifestations (3).

Brucellosis is usually asymptomatic in non-pregnant women , but pregnant women infected with *B. abortus* develop placentitis, which due to abortion in last period of pregnancy or stillbirth, Even in the absence of fetus infection, the bacteria shedding through the placenta to fetal fluids and vaginal discharges. The regional lymph nodes, liver and mammary gland can also be infected and bacteria can be excreted in milk. *Brucella* infection is mainly an occupational disease of laboratory worker, veterinarians, farmers, shepherds and meat inspector. (4). Brucellosis in cattle caused by *B. abortus*, which usually transmitted by contact with the placenta, fetal fluid and fetus from infected animals. cattle may shed the organism in milk and uterine discharges , shedding in milk can be intermittent or lifelong (5). In Iraq incidence of human brucellosis is increasing , and lack of awareness campaigns, illiteracy , policies or appropriate use of resources are contributing factors to this development (6). The aim of this study was to establish the seroprevalence of brucellosis in diary women seller and their families , and documented risk factors of human brucellosis and the potential reservoir of pathogens.

Materials and Methods

Samples collection

Blood samples were collected from women who sell dairy products (hawkers) in city markets and resident in different villages, furthermore as their families who lived as a contact with cattle and participated in make the dairy product. 186 (30 women +156 of their families) Blood samples were collected into silicon-coated tubes without anticoagulant for isolation of sera. The blood Sample centrifuged and the serum was extracted. After that (RBT) was used to determine the seropositivity. Briefly, drop of serum mixed with an equal volume of antigen , then The mixture is agitated gently for 4 minutes at ambient temperature, If an agglutination reaction occurred, the sample was recorded as a positive case (and negative otherwise) .

Molecular diagnosis

Genomic DNA was extracted

DNA was achieved using a genomic DNA purification kit (Invitrogen, Paisley, U.K.) according to the manufacturer's instructions.

.Throughout washing buffer 1 was

A 200µl blood samples were transferred to sterile 1.5ml microcentrifuge tube, and then added 20µl of proteinase K and mixed by vortex. After that, 200µl of binding buffer was added to each tube and mixed by vortex to achieve maximum lysis efficiency, and then all tubes were incubated at 60℃ for 10 minutes. A 100µl of isopropanol was added to mixture and mixed well by pipetting, and then briefly spin down to get the drops clinging under the lid. The lysate was carefully transferred into binding filter column that fitted in a 2 ml collection tube, and then closed the tubes and centrifuged at 8000 rpm for 1 minute . Throughout lysate was discarded in disposal bottle, and then 500µl washing buffer 1(W1) was added to each Binding filter column, and centrifuged at 8000 rpm for 1 minute discarded in disposal bottle, and then 500µl Washing buffer 2 (W2) was added to each binding filter column, and centrifuged at 8000 rpm for 1 minute .Throughout washing buffer 2 was discarded in disposal bottle, and then the tubes were centrifuged once more at 12000 rpm for 1 minute to completely remove ethanol. After that, GD binding filter column that contain genomic DNA was transferred to sterile 1.5ml microcentrifuge tube, and then added 50µl of Elution buffer and left stand the tubes for 5 minutes at room temperature until the buffer is completely absorbed into the glass filter of Binding column tube. Finally, all tubes were centrifuged at 8000 rpm for 1 minute to elute DNA, and storage at -20℃freezer.

Table(1): Specific genes for detection of *B.abortus* &*B. meltitensis*.

Genomic DNA Profile

The extracted genomic DNA was checked by using Nanodrop spectrophotometer (THERMO. USA), which measured DNA concentration (ng/µL) and checked the DNA purity by reading the absorbance at (260 /280 nm) as the following steps:

1-.After opening up the Nano drop software, chosen the appropriate application (Nucleic acid, DNA).

2- A dry chem-wipe was taken and cleaned the measurement pedestals several times. Then carefully pipette 2µl of free nuclease water onto the surface of the lower measurement pedestals for blank the system.

3-The sampling arm was lowered and clicked OK to initialize the Nano drop, then cleaned off the pedestals

1µl of blood genomic DNA was added to measurement.

Gene	Primers name	Primers 3' → 5'	Fragment size
BM EII04 66	Cy5-CCTCGGCATGGCCCGCAA -BHQ-2	TCGCATCGGCAG TTTCAA	112bp
BruAb2_0168	FAM-TGGAACGACCTTTGCAGG CGAGATC-BHQ-1	GCACACTCACCTTCCACAACAA	222bp

Real Time quantitative qPCR

The Real time PCRs for species differentiation were based on unique genetic loci of *B. melitensis* and *B. abortus*. The regions were chosen for the construction of primers and TaqMan® probes for species differentiation: BMEII0466 gene for *B. melitensis* and BruAb2_0168 gene for *B. abortus* (originally developed by (7). and this technique was carried out according to method described by (8) as shown in (Table-1). Real-Time PCR master mix was prepared by using (AccuPower® DualStarTM qPCR PreMix kit Bioneer. Korea), and done according to the company instructions. shown as in (Table-2).

Table (2): Real-time quantitative qPCRthermocycler .

Step	Condition	Cycle
Pre-Denaturation	**95 °C 5 min**	**1**
Denaturation	**95 °C 20 sec**	**50**
Annealing/Extension		
Detection (Scan)	**60 °C 30 sec**	

Serological diagnosis

Cattle Brucellosis

In order to determine the local prevalence of cattle brucellosis, records of brucellosis test results from villages animals were consulted. Randomly selected cows and buffalos (from the local population). Blood samples (20 cow and 20 buffalos in total) were collected from the jugular vein and serum was extracted and processed as described in the previous paragraph (RBT). then the positive sample cluttered on Brucella agar (Oxoid, UK) to detect *Brucella* species. Agars were incubated at 37°C for 8 days in a 5–10% carbon-dioxide incubator and read every 24 hours from day three of incubation for colony growth (9). Biochemical tests carried out by Api-20 E Kits.(BioMerieux).

Milk Ring Test

Twenty raw milk samples were collected from the milk main pot before boiling. The test was done by adding drop of antigen to milk sample (1 ml). The height of the milk column in the tube must be at least 25 mm. . then The mixture is agitated gently for (1 hour) at ambient temperature. A positive reaction is indicated by formation of a dark blue ring above a white milk column. The test is considered to be negative if the colour of the underlying milk remains homogeneously dispersed in the milk column(10)

Results and Discussion

Brucellosis prevalence in Dairy Women Seller

In this study was showed 6 (20%) out (30) women dairy seller were Seropositive whereas 24 (80%) seronegative result shown as (Table.3).

Table (3): Prevalence of brucellosis among dairy women seller (DWS)

Samples	number	Seropositive	Seronegative	Age of DWS
Dairy Women Selle	**30**	**6 (20%)**	**24(80%)**	**(22-45)**

Among the families of diary women seller the prevalence of seropositive 22/70(31.5%)

were women, 10/38(26%) men,11/58(19%) children,1/18(5.5%) babies less than 2 (Table. 4).

Table(4): Prevalence of brucellosis among families of dairy women seller (DWS)

Family	number	Seropositive	Seornegative	Percentage of infection
Women	**70**	**22**	**48**	**31.5%**
Men	**38**	**10**	**28**	**26%**
Children (2-12)	**58**	**11**	**47**	**19%**
Babies less than 2	**18**	**1**	**17**	**5.5%**
Total	**184**	**44(24%)**	**140(76%)**	

The current study, revealed a high prevalence (20%) of brucellosis among (DWS). This results was consistent with the results of(11) in which showed that the prevalence of brucellosis among pregnant women who contact with domestic animals (cattle, goat, or sheep) and consumption raw cow's milk were (25%). The most common transmission routes of *Brucella* infection in humans are through direct contact with infected livestock or consumption of

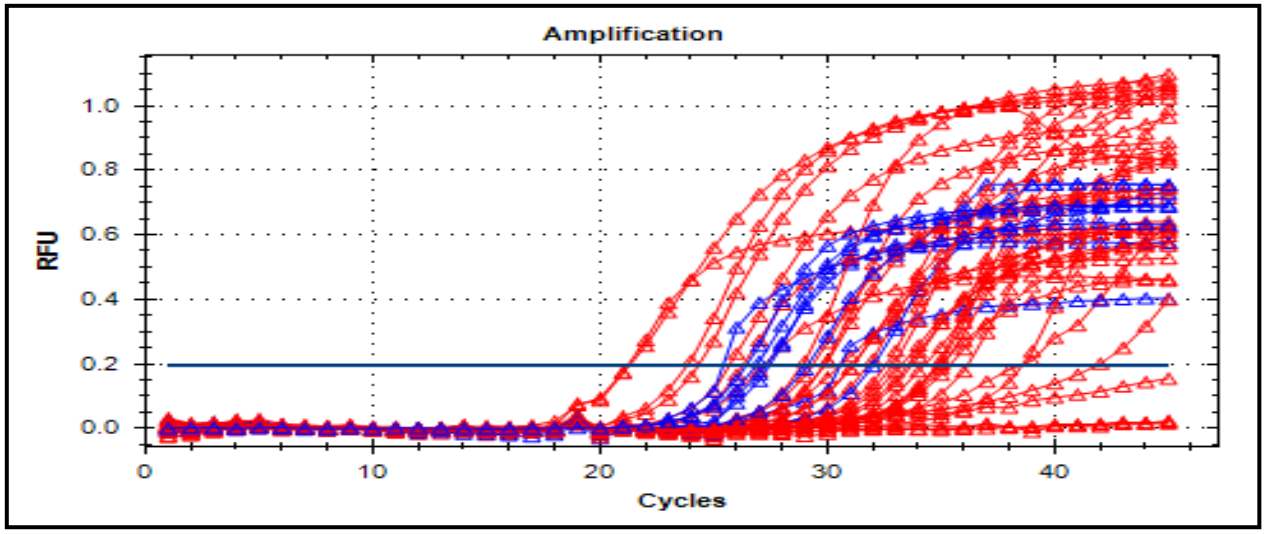
unpasteurized dairy products.(12).The prevalence of Brucella disease among the families of (DWS) were high in women followed by men then children. and this agreement with AL-Khafaji (6) in Iraq(Babylon province) in which reported the prevalence of brucellosis among women (66%) , children (1-5) (38%) and men were (33%). The infection of women brucellosis more than men , could explained that the majority of women were direct contact with milk and its products more than men and children(13).

Children infection due to they are more milk consumers especially fresh without pasteurization. While prevalence the infection among husbands of (DWS) and others men due to brucellosis may spread through sexual activity with carry women(14). The families of (DWS) may also be at risk as domestic exposure may be inseparable from occupational exposure when animals were kept in close proximity to living accommodation. In some villages , the animals are kept in the yards of homes and may even be brought inside, especially in winter season . In the case of recently aborted animals, this has resulted in infection of entire households. The use of dried dung as a fuel may also import infection into households. It should be noted that brucellosis often presents as clusters of cases in a family or tribal group, usually relating to a common infected food source, and often follows an outbreak in animals. kids can be particularly at risk as they may adopt newborn or sick animals as pets. All *Brucella* Seropositive (50) sample were tested by real time PCR reaction to detected *Brucella* species.

BMEII0466 gene for *B. melitensis* was distinguished in 27 (54%) sample , on the other hand , BruAb2-0168 gene for *B. abortus* were detected in 11 (22%) sample . (Fig.1) and (Table 5). Only (38) seropositive from (50) were positive to PCR reaction because of *Brucella* is intracellular and the most infected people are taken antibiotic before bacterial diagnosis(14).

Table(5):Diagnosis *Brucella* species by real-time qPCR reaction

Brucella seropositive	Negative For PCR reaction	Positive For PCR reaction	*B. meltitensis* (E110644 gen)	*B.abortus* (b-0168 gen)
50	12	38	27 (54%)	11(22%)



Fig(1): Real-Time PCR amplification plot of K-DNA region in *B. meltitensis* positive samples.

Figure (1): Real-Time PCR amplification plot of K-DNA region in *B. meltitensis* positive samples.

Our results were agreed with Debeaumont (15) they diagnosed human brucellosis by Real-time PCR reaction ,among 17 culture –proven brucellosis patients sera from 11 gave positive amplification signal , corresponding were to a sensitivity of 64.7%. Among the (20) women (6 WDS+14 of their families) were seropositive by (RBT), The questionnaire showed 10 (50 %) of them had aborted and 2 (10%) presented with a stillbirth (Table. 6). Also found in the current study, together

with findings by (4) gave insights into the otherwise controversial Brucella-associated abortion in humans, the association between abortion/stillbirth and brucellosis in humans is controversial (4). This could be due to absence of erythritol (a 4-carbon sugar alcohol which is the preferred carbon source for *Brucella*) in human placentas as opposed to ruminant placentas (16).

Table(6): Association between abortion/stillbirth and brucellosis in women.

Women Seropositive (RBT)	WDS	Women of WDS Families	Abortion	Stillbirth
*20	6	14	10	2

*The age between (22-40 years old)

Brucellosis prevalence in dairy cattle

The prevalence *Brucella* infection among the dairy cattle were not high , out of (20) cows (2) were seropositive compared with (2) buffalos from total (20) (Table .7). According to records from (Wasit veterinary hospital) the prevalence of brucellosis in cattle declined (15%-8%) from the year 2011-2014. Due to vaccination camping against cattle brucellosis and encourage the framers to use artificial insemination because cattle brucellosis spread through natural insemination

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Table(7): Brucellosis prevalence in dairy cattle .

Cattle	Number	Seornegative	Seropositive	Percentage
Cow	20	18	2	10%
Buffalos	20	18	2	10%

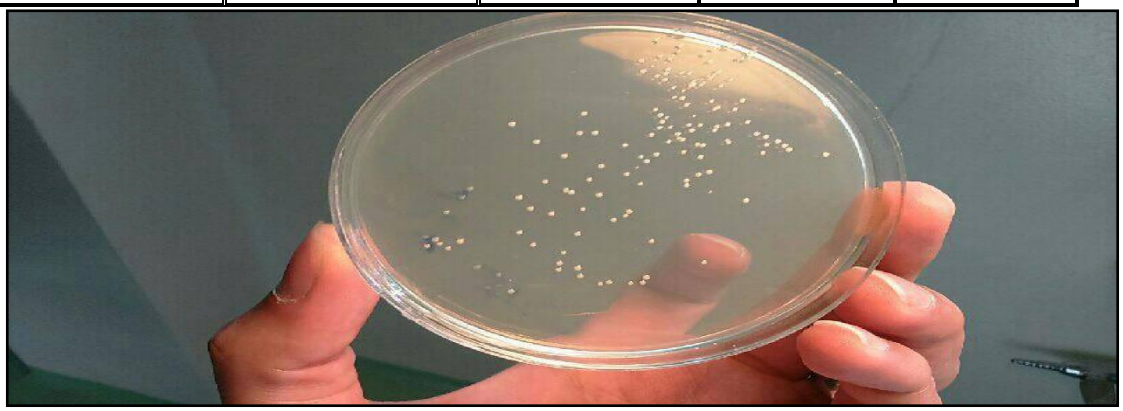

Figure (2): *Brucella abortus* on Brucella agar

***Brucella* prevalence in milk**

Forty raw milk samples 18 (45%) were positive for milk ring test (Table.6). Contaminated milk with bacteria may due to collect milk from all lactating cattle in one (pot). This results were agreed with (17) , They were isolated *Brucella* from eleven out of 107 milk samples from cattle in Uganda.

Brucella infected lactating female animals shed the bacteria in their milk since the organism relocates to the udder from the pregnant uterus upon delivery (18) .This has public health implications in the region since most of the milk is consumed unpasteurized. One third of the seropositive cattle were not shedding the *Brucella* in the milk suggesting that these animals either had cleared infection or were chronically sick (19).

Table (8). Milk ring test to detect contaminated milk with *Brucella*

Milk samples	Number	Positive	Negative	Percentage
Cow milk	20	10	10	50%
Buffalos milk	20	8	12	40%

Conclusions

1.Increasing the infection rate with brucellosis among DWS and their families in Wasit province

2. *B. mellitensis* is the most common cause of human brucellosis among WDS and their families.

3. probably association between abortion/stillbirth and brucellosis women.

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