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The Effect Of Brucella Melitensis In Milk And Dairy Products On Public Health And Food Safety And Evaluating The Effects Of Ph And Thermal Processing

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Abstract
Brucella melitensis is a pathogenic bacterium that responsible for brucellosis, a zoonotic disease of significant public health concern. This study aimed to isolate and identify B. melitensis from raw milk and dairy products from sheep and cattle by using classical and molecular techniques and to evaluate the effects of pH variation and cold storage on bacterial viability. In addition, the antibiotic resistance profile of the isolates. The Results showed that B. melitensis was present in several raw milk and dairy products samples (11%) and the percentage of isolates were higher in sheep (16.66) than in cattle (5.33%) and recorded significant differences in milk isolates than in other samples (cheese and yoghurt). Bacterial growth decreased significantly at 0°C and low pH levels, whereas moderate cold (4°C to 10°C) supported longer survival. Notably, the isolates exhibited resistance against azithromycin and streptomycin.
These findings highlight the persistence of Brucella. melitensis in milk and milk products of ewes and cows, and inappropriate conditions effect on bacterial growth of the these products during storage; controlling and monitoring of environmental factors that prevent bacterial survival and emphasize the need for strict food safety measures, including pasteurization and PH, and should apply antibiotic susceptibility test to detect bacterial antibiotic resistance.
Key words: *public health, B. melitensis, polymerase chain reaction, antibiotic susceptibility.*

INTRODUCTION

Brucellosis is a highly important zoonotic disease, with a global prevalence and a significant. diseases that causes threat to public health and economic implications), caused by bacteria of the genus Brucella, which are facultative intracellular organisms, and B. melitensis it has consider the most virulent of this genus(1) The disease is most commonly transmitted through the consumption of contaminated foods, such as unpasteurized milk and dairy products, or through contact with infected animals, making it common among veterinarians farmers, and slaughterhouse workers. Transmission can also occur in laboratory settings (2,3,4,5) Brucella strains are aerobic, but some require an atmosphere containing 5–10% carbon dioxide to grow.. During food processing and preparation, microorganisms are exposed to multiple environmental stresses, such as high and low temperatures, acids, and pressure, as well as preservatives and disinfectants. These factors weaken or eliminate microorganisms, limiting their proliferation and contributing to longer, safer food preservation. However, these stresses can lead to a phenomenon known as stress hardening, which occurs when microorganisms develop increased resistance to lethal environmental stressors after previously being exposed to a non-lethal stressor. This phenomenon has been documented in numerous microorganisms(6,7,8)Low temperatures are widespread used in food preservation to limit growth and survival to pathogenic microorganisms. even so, studies suggest that some microbes may adapt to low temperatures, giving them an enhanced ability to survive in similar or even adverse environments. This is known as the cold shock response (9,10)One of the most significant challenges in combating brucellosis is the development of antibiotic resistance, as well as the difficulty many effective antibiotics have in reaching the cells where Brucella multiplies. As a result, treating infections after they occur becomes complex and can lead to serious, long-term health complications if not effectively controlled (11)Agricultural area of Iraq, brucellosis is considered one of the most critical

bacterial diseases, primarily due to the consume of raw milk and by products, especially cheese (12), and according to what mentioned previously and in addition to insufficient information is available on response of Brucella melitensis to PH and cold treatments. Therefore, this study aimed to detection of Brucella infection by using PCR technique to milk and milk products samples, study the antibiotic susceptibility and evaluate the effect of PH and cold shock on the sensitivity of B. melitensis to various environmental stresses

MATERIALS AND METHODS

A total of 300 samples of milk and milk products were collected from cows and ewes from fields in Iraq; The samples were kept under 4°C immediately after collection and then transported to the Research Unit at Medicine Laboratory in the College of Veterinary, University of Karbala (Iraq) within two hours to ensure sample integrity

Bacterial Culture and Biochemical Testing:

In the laboratory, samples were cultured using a medium specifically designed for Brucella growth. Samples were inoculated onto agar plates containing 7% sheep blood (Brucella agar) (HIMEDIA . India)The plates were incubated at 37°C in carbon dioxide atmosphere for 5–7 days. After colonies grew, biochemical tests were performed to identify the isolates .based on (13)

Molecular Analysis (PCR):

DNA of Bacteria was extracted using a Bacterial Genomic DNA Extraction Kit (Geneaid , Taiwan) to confirm the molecular identity of the isolates

. Following extraction, polymerase chain reaction (PCR) was implemented using specific primers to detect Brucella species. The following primers were used:

- **Forward primer: 5'-GGCGTGTCTGCATTCAACG-3'**
- **Reverse primer: 5'-GGCTTGTCTGCATTCAAGG-3'**

PCR products were analyzed to determine the identity of the isolates based on the molecular result.(14)

Antibiotic Sensitivity Test:

Freshly grown pure colonies of Brucella isolates were selected. The colonies were then suspended in sterile saline until a turbidity of 0.5 according to the McFarland standard was reached (approximately 1.5 x 10^8 CFU/ml). The turbidity of the suspension was checked using a turbidity meter, with visual comparison to a standard McFarland tube. The bacteria were cultured using Mueller-Hinton agar. The bacterial suspension was evenly distributed over the entire surface of the agar using a sterile swab to ensure the formation of a homogeneous layer of bacterial growth. Antibiotic discs were then applied: Using sterile forceps, the selected antibiotic discs were gently placed onto the surface of the culture medium, ensuring that the discs were spaced far enough apart to avoid overlapping inhibition zones. The plates were incubated at 37°C. Incubation was carried out in an environment containing 5-10% carbon dioxide (CO_2). Incubation lasted for 10 min. 48 consecutive hours .After the incubation period, the diameters of the zone of inhibition around the antibiotic discs were measured using a fine ruler. A group of common antibiotics was selected to treat Brucella (e.g.,, Azithromycin (15 µc g) Amikacin(10 µg) ,
gentamicin (10 µg), streptomycin (10 µg), rifampin (30µg), doxycycline (30 µg), ceprofloxacine(10 µg), **(15)**

PH experimental study

pH measurements were performed in this study to evaluate the optimum pH for Brucella growth according to other study (16),

Sufficient amounts of Brucella melitensis suspension of bacteria were prepared to achieve a final concentration of approximately 1 × 10^4 CFU/mL in all experimental samples. Tryptic Soy Broth (TSB) tubes of different pH levels, as well as dairy products including milk, yogurt, and cheese, were used for bacterial inoculation. All procedures were performed aseptically within a biosafety cabinet to prevent contamination. For TSB broth, sterile tubes containing 9 mL of TSB were inoculated with 1 mL of bacterial suspension, ensuring a final concentration of 1 × 10^4 CFU/mL. The tubes were gently mixed to allow uniform distribution of bacteria and incubated at 37°C in a 5% CO_2 environment, protected from light.For milk, 9 mL of pasteurized milk was transferred into sterile tubes and inoculated with 1 mL of

bacterial suspension. The tubes were gently inverted several times to mix the contents before incubation under the same conditions as the TSB tubes. For yogurt, 9 g of sterile yogurt was dispensed into sterile tubes using a sterile spatula or pipette. After the addition of 1 mL of bacterial suspension, the samples were thoroughly mixed using a vortex mixer or a sterile spatula to ensure homogeneity. The inoculated tubes were incubated at 37°C with 5% CO_2, protected from light. For cheese, 9 g of cheese was weighed and placed into sterile tubes. To facilitate bacterial distribution, 1 mL of sterile saline was added along with 1 mL of bacterial suspension. The mixture was homogenized under sterile conditions before incubation at 37°C in a 5% CO_2 environment. Brucella broth was prepared and adjusted to six various pH levels: 4.5, 5.5, 6.0, 7.0, 8.0, and 9.0 and at predetermined time points (3, 7, and 14 days), 1 mL (or 1 g for solid samples) of each inoculated sample was withdrawn under aseptic conditions. The pH of each sample was measured immediately using a pH meter. Observations regarding physical changes in the dairy products were recorded the. growth was assessed by examining colony formation on Brucella agar at three time points. Colony morphology and density were recorded for comparative analysis. The growth of Brucella melitensis at each pH level was analyzed based on the presence or absence of visible colonies and their characteristics. Bacterial Suspension Preparation

Statistical l Analysis:

Data were analyzed using SPSS .The chi-square test was used to examine the relationship between categorical variables (dairy product type and Brucella melitensis presence).

To assess bacterial survival at low temperatures,

additional inoculated samples were stored at 0°C, 4°C, and 10°C. All samples were protected from light and monitored at specific time intervals.

At 0°C, 4°C, and 10°C, samples were withdrawn after 3 days, 7 days, and 14 days. At every time point, 1 mL (or 1 g for solid samples) of each inoculated sample was removed under aseptic conditions(17)

RESULTS

The total of 300 samples of (cow and ewes) were examined by using traditional (culture) and molecular methods (PCR) (fig: 1) to detect and identify Brucella infection, the result showed 33 (11%) were positive and the ewes samples recorded positive isolates more than cow samples; while the significant differences between milk and milk products indicating an association between sample type and infection rate as mentioned in the table (1).

animal	Type of sample	No. of sample	Positive	%	Negative	%	Statistical analysis
Cattle	Milk sample	100	6	6	94	94	X^2=1.84 DF= 2 P value= 0.39
	Cheese sample	25	2	8	23	92	
	Yoghurt sample	25	0	0	25	100	
	total	150	8	5,3	142	94,6	
sheep	Milk sample	100	22	22	78	78	X= 6.28 DF= 2 P value= 0.043
	Cheese sample	25	2	8	23	92	
	Yoghurt sample	25	1	4	24	96	
	Total	150	25	16.6	125	83.3	

No of sample	Antibiotic	Disc Concentration (µg)	Sensitive%	Resistant%	Intermediate%
33	Azithromycin	15(µg)	39,39	61%	0%
33	Streptomycin	10(µg)	6.1%	15.1%	0%
33	ciprofloxacin	5(µg)	81.8%	18.1%	0%
33	Doxycycline	30(µg)	96.9%	3%	0%
33	rifampicin	5(µg)	81.8%	9%	9%
33	gentamicin	(10 µg)	96.9	3	0
33	amikacin	30(µg)	96.9	3	0

- **None of the yogurt samples from cows showed any positive results.**
- **Statistical analysis showed a significant difference in infection rates between ewe and cows**

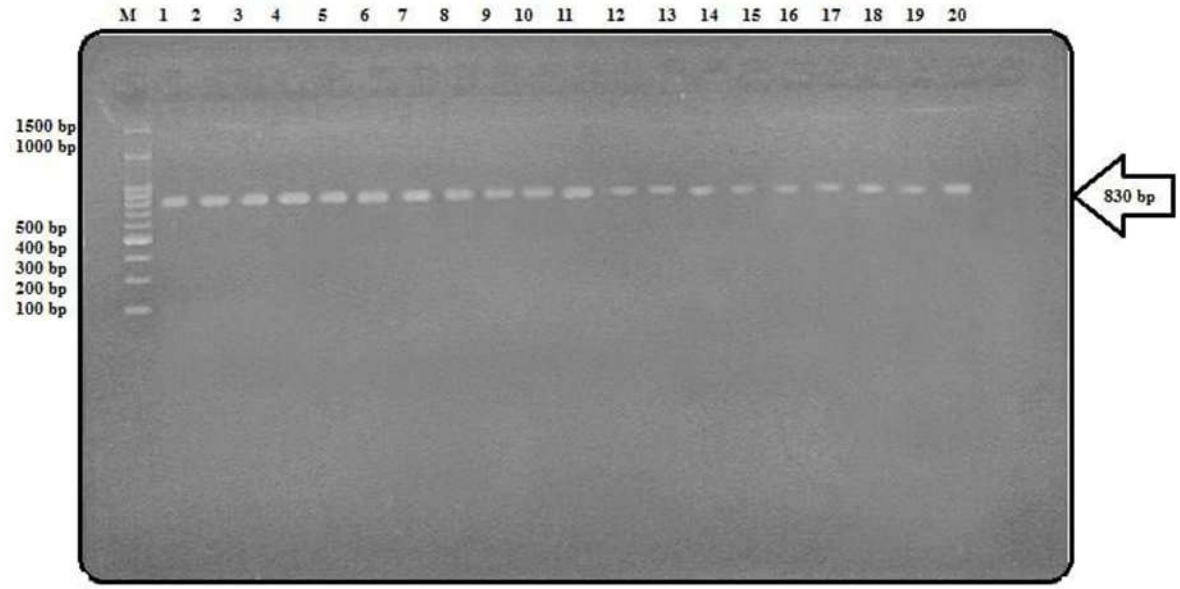

Figure 1: Agarose gel analysis of PCR products, the lane (M) represent the DNA ladder; lanes numbers 1 to 20 represent 830 bp for Brucella melitensis

The bacterial resistance has yielded worrying results during the antibiotic susceptibility test due to a number of bacteria were resistance or moderately resistance to some antibiotic as in table (2).

Effect of pH on Brucella

Effect of pH on the growth of Brucella melitensis in dairy products The study showed that pH has a direct and decisive effect on the growth of Brucella melitensis in different dairy products (milk, yogurt, cheese): In milk at pH 7.0 very good bacterial growth was recorded, with numbers on day 3 reaching approximately 1×10^7 CFU/ml, peaking on day 7 at 4×10^7 CFU/ml, and then gradually declining to 1×10^7 CFU/ml on day 14. At pH 6.5: There was also significant growth but less than pH 7, with counts on day 3 reaching about 1×10^6 CFU/ml, and on day 14 declining to 1×10^5 CFU/m l .At pH 5.5: Limited growth was observed; the bacterial count on day 3 was approximately 1×10^5 CFU/ml, and decreased dramatically until it was almost gone on day 14. At pH 4.5 or less no bacterial growth was observed., indicating that this acidity is lethal to them. In yogurt had a naturally low pH (< 4.5).Very little growth was observed in

the first 72 hours (approximately 1x 10^3 CFU/ml), but after 14 day no growth was detected, which means that the acidic environment in the milk prevents long-term survival of the bacteria. In cheese At pH 7.0 and 6.5: The highest growth rates were recorded. Counts on day 3 were approximately 1 × 10^7 CFU/ml, increased to 5 × 10^7 CFU/ml on day 7, and then decreased to 1 × 10^7 CFU/ml on day 14. At pH 5.5: The bacterial count on day 3 was about 4 × 10^5 CFU/ml, and decreased to about 1 × 10^4 CFU/ml on day 14

Table (3)show the effect of PH on growth of Brucella bacteria in dairy product

Product	pH	Day 3 (χ^2=15.3, p=0.0092)	Day 7 (χ^2=15.3, p=0.0092)	Day 14 (χ^2=15.3, p=0.0092)
Milk	4.5	Nil	Nil	Nil
	5.5	Growth 1 X 10^4 CFU	Growth 1 X 10^4 CFU	Growth 1 X 10^4 CFU
	6.5	Growth 1 X 10^6 CFU	Growth 1 X 10^6CFU	Growth 1X 10^5 CFU
	7	Growth 1 X 10^7 CFU	**Growth** 4 X 10^7 CFU	**Growth** 2 X 10^6 CFU
	8	**Nil**	**Nil**	**Nil**
	9	**Nil**	**Nil**	**Nil**
Yogurt	4.5	Nil	**Nil**	**Nil**
	5.5	Nil	**Nil**	**Nil**
	6.5	Growth 1 X 10^7 CFU	**Growth** 1X 10^7 CFU	**Nil**
	7	Growth 1 X 10^3 CFU	**Nil**	**Nil**
	8	**Nil**	**Nil**	**Nil**
	9	**Nil**	**Nil**	**Nil**
Cheese	4.5	Nil	**Nil**	**Nil**
	5.5	Growth 4 X 10^5 CFU	Growth 1 X 10^4 CFU	Growth 1 X 10^4 CFU
	6.5	Growth 1 X 10^6 CFU	Growth 5X 10^5 CFU	Growth 1 X 10^7 CFU
	7	**Growth** 1 X 10^7 CFU	**Growth** 5X 10^7 CFU	**Growth** 1 X 10^7 CFU
	8	**Nil**	**Nil**	**Nil**
	9	**Nil**	**Nil**	**Nil**

Growth Data at Low Temperatures

The influence of temperature on the growth of Brucella has a varied response with different temperatures when kept in a cold environment under (10, 4 and 0°C) during days (3, 7 and 14) as mentioned in table (3).

Growth Data at Low Temperatures:			
Temperature	**Day 3**	**Day 7**	**Day 14**
0°C	**Growth**	**No Growth**	**No Growth**
4°C	**Growth**	**Growth**	**Growth**
10°C	**Growth**	**Growth**	**Growth**

DISCUSSION

Brucella melitensis widely distributed around the world and considered one of the important challenges that faced the food safety and public health; many studies were applied in Iraq to detect the Brucella prevalence and distribution (18). The Results showed the dairy product carry the risk of transmitting this bacteria to human which appeared the prevalence in sheep more than in cow, and it is considered the

main cause of brucellosis in human and This is mimic the results that showed in other studies with the finding that the prevalence of B. melitensis was highest in sheep compared to cattle(19). sheep milk and it products are represent source of infection of B. melitensis which is considered the most virulent to people according to reports from the the World Health Organization (20)The positive results that noticed during the present study that recorded by PCR test which approved by many studies one of the most accurate and fast technique used to detect and identify Brucella infection due to the highly sensitivity and specificity which distinguish this test(21,22) According to the WHO treatment guidelines(23), doxycycline with rifampicin is recommended as a long-term treatment plan for Brucella. It is important to conduct susceptibility testing before initiating treatment, especially given that numerous studies have shown Brucella to be increasingly resistant to some common antibiotics, such as rifampicin and trimethoprim. In some cases, Brucella resistance to azithromycin has reached 100%, a rate similar to that reported in China, suggesting the potential for the development of complete resistance to this drug in the near future. In Egypt, the rate of resistance to ciprofloxacin has been observed to be higher than that to rifampicin. Despite this serious trend, research into the mechanisms of Brucella antibiotic resistance remains limited (24,25), The results clarify that the milk isolates were more than the milk products from both ewes and cows suggesting that the physical and chemical factors during the milk products preparation may contribute to inhibit and limit the bacterial growth, in addition the milk directly taken from the animal without any processes (26) The results showed that temperature plays a pivotal role in regulating bacterial growth during the 14-day incubation period. Limited growth was observed at 0°C only on the third day, followed by a complete absence of growth on subsequent days, suggesting that extreme cold may impede metabolic processes and limited survival of bacteria. In contrast, bacterial growth continued at 4°C and 10°C throughout the study, indicating that these temperatures are more favorable for the growth of psychrotrophic bacteria.(17) The ideal pH range for growth is between 6.6 and 7.4, with a buffer medium with a pH close to 6.8 required to ensure optimal growth. As for temperature, optimum conditions range from 36 to 38.8°C, although some strains can grow in a range extending from 20 to 40.8°C; Yogurt's ability to inhibit the growth of many pathogenic microorganisms is attributed to the presence of active lactic acid bacteria (LAB), which produce lactic acid during fermentation. This acid lowers the pH of the medium, creating an acidic environment unfavorable for the growth of many acid-sensitive bacteria such as Brucella spp. In addition to the direct inhibitory effect of lactic acid, LAB also has the ability to produce antibacterial compounds such as bacteriocins, which enhances its Anti pathogenic effect.(27,28) Furthermore, the heat treatment process milk undergoes during yogurt manufacturing contributes to the elimination of pre-existing Brucella bacteria, reducing the possibility of transmission through dairy products(29)

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