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Application of conventional and molecular methods in the diagnosis of *Brucella melitensis* in ewes and cows from Karbala and Babylon Provinces

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***Abstract*— Brucellosis is one of the zoonotic bacterial disease that poses a threat to public health. It is transmitted to humans through the consumption of unpasteurized milk and dairy products. This study aims to detect the presence of *Brucella* bacteria in the samples of milk and by products (yogurt and cheese) taken from cows and ewes in Karbala and Babylon governorates. This study used specialized culture media and gene sequencing technology to extreme precision to identify bacterial species and biochemical tests for first diagnosis. The study started from October /2024 until April/2025 , 300 samples of milk and milk products (yogurt and cheese) were collected from cows and ewes The results showed varying in rates of infection between the two governorates, with the highest infection rate recorded in the Babylon governorate compared to Karbala. Gene sequencing results also proved that all positive isolates were of the *Brucella melitensis* species, that most common cause of infection in humans. This study confirms as the real risk of disease transmission through the consumption of unpasteurized local dairy products, highlighting the importance of health monitoring and early diagnosis to limit the spread of the infection**

Keywords —*: *Brucella melitensis* ,*gene sequencing* , *Karbala

INTRODUCTION

Brucella melitensis bacteria is the most important zoonotic pathogens that cause brucellosis, a major health and the economic loses in developing countries, including Iraq**(1).** *Brucella* is characterized by its high ability to survive within phagocytic cells, making it capable of causing chronic infection and resistant to many immune defense mechanisms ***(2)***

The most common Brucella species pathogenic to humans include *Brucella melitensis*, *B. abortus*, and *B.suis. B. melitensis* is the most aggressive and associated with human infections in Middle Eastern countries ***(3*)**. Infection is most often transmitted to humans through the consumption of contaminated animal products or through direct contact with infected animals, making disease monitoring and early diagnosis vital for control. Due to *Brucella* is very difficult to isolate in the laboratory due to its slow growth and specific nutritional requirements, selective culture media such as antibiotic-enriched *Brucella* agar used to isolate strains ***(4).*** Following isolation, standard biochemical tests such as catalase, oxidase, and urease are performed to help identify the species and strain ***(5).***

With the development of genomics, genomic sequencing has become a crucial tool for the accurate diagnosis and classification of *Brucella* strains, through the analysis of conserved genes bcsp31, ***(6).*** This type of analysis contributes to tracking patterns of epidemic spread and understanding the evolutionary relationships between local and global strains , **The study aimed to** isolate and identify *Brucella* bacteria from milk and dairy product samples (cheese and yogurt) taken from cows and ewes in Karbala and Babylon governorates, and to use special culture media to cultivate the bacteria and confirm their presence using a traditional laboratory method as well as to apply gene sequencing technology to accurately identify the *Brucella* species and strain

MATERIALS AND METHODS

Sample collection: During this study, about 300 samples of milk and milk products were collected from cows and ewes from fields in Karbala and Babylon ; 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

Isolation & identification ;Bacterial isolation using *Brucella* Agar 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**. (*7*)**

Biochemical Identification Tests: After colonies grew, A series of tests were performed on the suspected colonies to determine their biochemical identity, including (Catalase test, Oxidase test, Indole test, Urease test, and Triple sugar iron test **(8).**

Genetic analysis (Molecular Confirmation)

Sample Preparation for Whole Genome Sequencing: Pure bacterial colonies were selected and cultured in Brain Heart Infusion Broth. After incubation, the bacterial pellets were collected by centrifugation at 16,000 rpm for 1 minute. Bacterial DNA was extracted and its concentration measured using a Nanodrop device (Thermo Fisher), reaching a DNA concentration of 174 ng/µL in a total volume of 90 µL.Sample Submission and Whole Genome Sequencing: The extracted DNA samples were sent to Macrogen (Korea) for whole genome sequencing using the Sanger technique.**(9)**

Statistical analysis: SPSS was used to analyze the data. The statistical significance level was considered at ($p < 0.05$

RESULT & DISCUSSION

(300) samples of milk and dairy products taken from cows and ewes in the Karbala and Babylon governorates were analyzed. The results showed the 37 samples, **(30%)** showed growth of bacteria suspected to be Brucella on Brucella Agar. Cultivation results on Brucella Agar showed the growth of distinct bacterial colonies shape after an incubation period of 48 to 72 hours at 37°C. The colonies were too small, round , smooth-surfaced, shiny, and they had a light honey-transparent color—morphological characteristics consistent with those described for *Brucella melitensis*. **(9)**

The appearance of these characteristics in a selective medium is an important initial step in diagnosis, as *Brucella* Agar contains components that promote the growth of *Brucella* and inhibit the growth of associated bacteria, facilitating the isolation process. These results were later supported by biochemical tests and gene sequencing, which confirmed that the isolates obtained were *Brucella melitensis*.

These results indicate that the use of *Brucella* medium is effective in the initial detection of *Brucella* infection in samples taken from milk and milk products. It also highlights the importance of using specialized laboratory culture in epidemiological diagnostic studies of *Brucella*, especially in areas where unpasteurized dairy products are consumed **(10)**

A total of 300 samples were collected, 150 from Babylon Governorate and 150 from Karbala Governorate, to confirm the presence of *Brucella* bacteria. The results showed that 25 samples tested positive in Babylon, with a positivity rate of approximately 16.6%, while only 12 samples tested positive in Karbala, with a positivity rate of approximately 8%. These results indicate that the incidence of brucellosis in Babylon Governorate is higher than in Karbala Governorate**.**

Table 1. show the distribution of isolation sample between two provinces Karbala and Babylon

The provinces	No. of sample	Positive	%	negative	%
Babylon	150	25	16.6	125	83
Karbala	150	12	8	138	92
Total	300	37	12.3	263	87.6
Statistically analysis	X^2= 5.21, DF= 1; P=0.0224				

Significant differences (P< 0.05), more prevalence in Babylon than Karbala Governorate.

The difference in brucellosis rates between Babylon and Karbala can be attributed to multiple factors, including dietary behaviors, livestock husbandry practices, geographic distribution, and challenges in prevention programs. Therefore, it is recommended to strengthen health awareness programs, improve animal husbandry practices, and provide the necessary vaccines to limit the spread of the disease in the most affected areas**.(11,12).**

Table 2. Identification of *Brucella melitensis* by biochemical tests.

Test	Result
Oxidase	+ve
Indole	-ve
Catalase	+ve
Urease	+ve
Citrate	-ve
TSI	-ve

The results of the biochemical tests were consistent with the known characteristics of *Brucella melitensis*. Rapid urease activity is one of the most important indicators that help distinguish it from other Gram-negative
bacteria. Furthermore, the positive results in the oxidase and catalase tests support the obligate aerobic nature of this bacterium.

The negative results in the citrate, indole, and TSI tests are consistent with describing *B. melitensis* as a bacterium that does not utilize sugars or proteins through gaseous fermentation and does not produce sulfur compounds, making it easier to distinguish it from other bacterial species such as *Salmonella or E. coli*. **(13)**

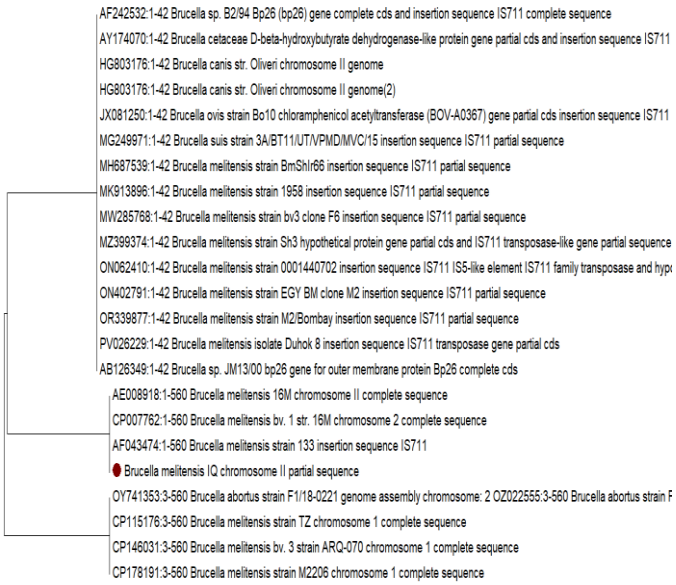

Figure 1. The structure of the evolutionary tree, which consists of branches, nodes, families, and distinct taxa, reveals the genetic similarities and differences between the studied lineages.

The Iraqi *Brucella* strain is found on a branch with a solid trunk, indicating a close genetic relationship with other globally isolated *Brucella* strains. This arrangement indicates that the Iraqi strain shares a more recent common ancestor with these strains, especially those of international origin such as the US, UK, and China, compared to other *Brucella* species such as *Brucella ovis*, *Brucella suis*, and *Brucella canis*, which appear on more distantly related branches The Iraqi strain clusters within a major clade that includes strains with complete genome sequences, demonstrating a high degree of genetic conservation within *Brucella melitensis*. The location of the root of the tree, closest to species such as *Brucella suis and Brucella canis*, reflects the basal lineage from which the recently branched Brucella melitensis lineages evolved. Multiple internal nodes are formed, representing hypothetical common ancestors linking closely related lineages. Each node produces branches terminating in taxa, each representing a single *Brucella* isolate or strain. The presence of the Iraqi isolate near a common node with strains from different countries indicates a regional distribution and limited genetic variation among these isolates. The tree structure shows that, although global *Brucella melitensis* strains are highly conserved, there is slight genetic variation, likely due to geographic and environmental factors. The Iraqi lineage does not constitute a unique or highly divergent branch, meaning it has not undergone significant genetic differentiation from globally distributed lineages. Overall, the phylogenetic position of the Iraqi *Brucella melitensis* strain provides evidence of its genetic relationship with globally circulating strains, reinforcing the importance of international surveillance and genomic monitoring for understanding the epidemiology of brucellosis. In conclusion: Strengthening veterinary health surveillance in Babylon Governorate, given the high percentage of positive bacterial isolates (25 out of 150) compared to Karbala (12 out of 150), suggesting the potential presence of environmental or administrative factors contributing to the spread of infection. **(15,16)**

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