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Conventional and Molecular Identification of *Brucella* spp. in Different Farm Animals from Different Rural Areas in Wasit Province, Iraq

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Abstract
The present study was conducted to identify the occurrence of *Brucella* spp. in different farm animals from different rural areas of Wasit Province, Iraq. The study involved in the sampling of 165 blood samples from 81 cows, 50 buffalos, 23 sheep, and 11 goats. These samples (5 ml/each) were subjected to Rose Bengal test (RBT), regular bacterial cultivation and identification, positive-RBT-dependent real-time PCR (RT-PCR), and cultivation-dependent conventional *16S rRNA* gen-and *B5/B4* gene-based PCR, which were followed by a phylogenetic investigation utilizing partial gene sequencing. The results of the RBT revealed the presence of *Brucella* antibodies in 14 animals, which were distributed as 5 (6.2%), 1 (2%), 3 (13.04%), and 2 (18.2%), respectively. The findings showed significant (p<0.05) correlation of infection with age, sex, and geographical areas in all animals. The cultivation demonstrated the presence of *Brucella* spp. in 6 (42.9%) of the RBT positive samples, which were confirmed in RT-PCR and conventional PCRs. The phylogenetic work revealed close similarity with isolates from India, Egypt, China, and USA. The present study results shows that *Brucella* spp. was present in cows, buffalos, sheep, and goats, which might be considered as important infection sources to spread the infection to different animal herds and areas.
Keywords: Abortion, Brucellosis, Stillbirth, Stormy abortion

Introduction
Brucellosis is a prevalent zoonotic disease, with an annual incidence of 500,000 cases. The illness was previously referred to as Malta fever or Mediterranean fever. Out of the various species of Brucella that have been identified, *B. melitensis, B. abortus,* and *B. suis* are the most significant in causing human diseases, while *B. canis* is the least significant. Thus far, there have been no documented cases of any disease being attributed to *B. ovis* and *B. neotomae* (1, 2). Brucellosis exhibits a diverse array of clinical symptoms, often persisting for varying durations ranging from a few days to many years. In people, the disease is seldom lethal but typically causes significant impairment. The spread of the disease occurs through multiple routes, such as the digestive system, pulmonary system, the mucous membranes, the skin, and coming into contact with bodily secretions. The primary mode of spread of illness from animals to humans is through the ingestion of uncooked meat and dairy products.

Brucellosis is frequently misdiagnosed, resulting in extended illness and insufficient treatment. Furthermore, the symptoms of the disease lack specificity, posing challenges in the diagnostic process (3, 4). The current epidemiological status of brucellosis in vulnerable animals in a country or area is an important factor in determining the appropriate diagnostic testing approach. Diagnostic tests serve multiple purposes, such as confirming a diagnosis, conducting screenings or epidemiological investigations, and verifying the presence of a disease. In nations where efforts are being made to eliminate brucellosis, it is crucial to have vigilant monitoring systems in place to avoid the recurrence of the disease via the importation of livestock or livestock products that are infected. The accuracy of diagnostic tests, particularly in the context of wildlife, remains a matter of concern (5). The identification of brucellosis is performed through direct approaches, such as bacteriological and molecular techniques, as well as indirect procedures, including vivo allergic approaches. The definitive method for detecting this illness is direct bacteriological evaluation, specifically the culturing of Brucella obtained from bodily fluids or tissues. Nevertheless, in order to overcome the challenges associated with bacteriological examination, molecular biological approaches, such as PCR, are being effectively employed to distinguish various Brucella species. Serological tests play a crucial role in diagnosis, although they can pose challenges in terms of interpretation. These techniques are employed for the primary diagnosis of brucellosis and also for monitoring the progress of treatment. Periodic serological readings are often advised due to the high occurrence of inaccurate negative serological tests during the initial stages of infection, as well as their ability to accurately diagnose and track the response to treatment (6, 7). The diagnostic methods for brucellosis include the RBT, RT-PCR, and complement fixation test (CFT). Nevertheless, none of these tests can singularly identify all distinct phases of brucellosis. Hence, a combination of tests can be utilized to achieve a conclusive diagnosis (9). The present study was conducted to identify the occurrence of *Brucella* spp. in different farm animals from different rural areas of Wasit Province, Iraq.

Materials and methods

Samples

The study involved in the sampling of 165 blood samples from 81 cows, 50 buffalos, 23 sheep, and 11 goats. These samples (5 ml/each) were subjected to RBT, regular bacterial cultivation and identification, positive-RBT-dependent real-time PCR (RT-PCR), and cultivation-dependent conventional *16S rRNA* gen-and *B5/B4* gene-based PCR. The samples were transferred to a microbiological lab, Al-Kut City, Iraq.

Rose Bengal Test

The test was done using guidelines from the kit purchased from bioMerieux, France. In which, a clear white-background plate was used.

Bacterial cultivation and biochemical tests

The methods depended on the used of cultivation on different primary and selective agar media. The grown colonies were examined for Gram's and Modified Ziehl-Neelsen’s stains and biochemical tests.

Extraction of DNA

The Wizard Genomic DNA purification kit (Promega, USA) was recruited and depending on the kit protocol. A NanoDrop was utilized to evaluate the obtained DNA.

Real-Time PCR

The Bosphore *Brucella* Detection kit V1 was employed using the 50 reactions as indicated by the kit. The reaction included 688µl PCR mix, 83µl detection mix1, 15µl detection mix2, 15µl internal control, 44µl positive control, and 500 µl dH_2O. The kit primers are not shown by the company due to patent purposes. The thermal conditions were 94°C-60s denaturation, 55°C-60s annealing, 72°C-60s extension, and 72°C-7mins final extension.

Conventional PCR

The primers employed are displayed on (Table 1). These primers were followed from Baily et al. (10).

Table 1: Primers recruited in the presentwork

Region	Nucleotide sequences	Size of PCR product (bp)
B4	-'5 TGGCTCGGTTGCCAATATCAA-'3	223 of a31-kDa *Brucell*-specific gene of membrane protein
B5	-'5 CGCGCTTGCCTTTCAGGTCTG-'3	
F	AGAGTTTGATCCTGGCTCAG	
R	GGTTACCTTGTTACGACTT	1500 16SrRNA

The PCR system was from (Promega, USA). It contained 5 units/µl DNA polymerase, 100 mM KCl, 0.1 mM EDTA, 10mM each of dNTPdATP. The agarose gel (2%) was run using an electrophoresis method, and the products were visualized utilizing a UV-based imager.

Phylogenetic analysis

The PCR positive productive were sequenced and the data were processed using NCBI-websites and MEGA X software for the construction of the phylogenetic tree.

Results

The results of the RBT revealed the presence of *Brucella* antibodies in 14 animals, which were distributed as 5 (6.2%), 1 (2%), 3 (13.04%), and 2 (18.2%), respectively. The findings showed significant (p<0.05) correlation of infection with age, sex, and geographical areas in all animals (Figure 1, Tables 2 - 13).

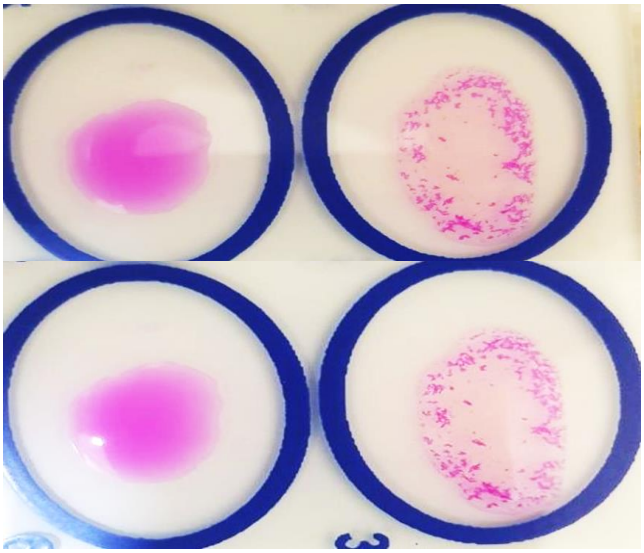

Figure 1: ***Brucella*-based Rose Bengal Test from ruminant-originated blood samples. Right column (pink granules): Positive reaction**

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Table 2: *Brucella* infection rate in cows according to age

Age range (Months)	No.	Infected No. (%)	*P* value
6-12	13	0	=0.00001*
13-24	59	5 (8.5)	
25-36	9	0	
Total	81	5 (6.2)	

* Significant correlation with 13-24 months of age

Table 3: *Brucella* infection rate in cows according to sex

Sex	No.	Infected No. (%)	*P* value
Males	24	1 (4.17)	=0.001*
Females	57	4 (7.02)	
Total	81	5 (6.2)	

* Significant correlation with sex (females)

Table 4: *Brucella* infection rate in cows according to geographical distribution

Name of village	No.	Infected No. (%)	*P* value
Yafa	40	3 (7.5)	=0.01*
Jyzaniyah	15	2 (13.3)	
Hakeem-2	13	0	
Besrugiyah	13	0	
Total	81	5 (6.2)	

* Significant correlation with geographical areas

Table 5: *Brucella* infection rate in buffalos according to age

Age range (Months)	No.	Infected No. (%)	*P* value
6-12	6	0	=0.00001*
13-24	33	1 (3.03)	
25-36	11	0	
Total	50	1 (2)	

* Significant correlation with 13-24 months of age

Table 6: *Brucella* infection rate in buffalos according to sex

Sex	No.	Infected No. (%)	*P* value
Males	4	0	=0.00001*
Females	46	1 (2.2)	
Total	50	1 (2)	

* Significant correlation with sex (females)

Table 7: *Brucella* infection rate in buffalos according to geographical distribution

Name of village	No.	Infected No. (%)	*P* value
Yafa	33	1 (3.03)	=0.01*
Jyzaniyah	10	0	
Hakeem-2	4	0	
Besrugiyah	3	0	
Total	50	1 (2)	

* Significant correlation with geographical areas

Table 8: *Brucella* infection rate in sheep according to age

Age range (Months)	No.	Infected No. (%)	*P* value
6-12	9	0	=0.001*
13-24	12	2 (16.7)	
25-36	2	1 (50)	
Total	23	3 (13.04)	

* Significant correlation with 25-36 months of age

Table 9: *Brucella* infection rate in sheep according to sex

Sex	No.	Infected No. (%)	*P* value
Males	2	0	=0.00001*
Females	21	3 (14.3)	
Total	23	3 (13.04)	

* Significant correlation with sex (females)

Table 10: *Brucella* infection rate in sheep according to geographical distribution

Name of village	No.	Infected No. (%)	*P* value
Yafa	8	2 (25)	=0.001*
Jyzaniyah	6	1 (16.7)	
Hakeem-2	6	0	
Besrugiyah	3	0	
Total	23	3 (13.04)	

* Significant correlation with geographical areas

Table 11: *Brucella* infection rate in goats according to age

Age range (Months)	No.	Infected No. (%)	*P* value
6-12	8	0	=0.0001*
13-24	2	2 (100)	
25-36	1	0	
Total	11	2 (18.2)	

* Significant correlation with 13-24 months of age

Table 12: *Brucella* infection rate in goats according to sex

Sex	No.	Infected No. (%)	*P* value
Males	2	0	=0.00001*
Females	9	2 (22.22)	
Total	11	2 (18.2)	

* Significant correlation with sex (females)

Table 13: *Brucella* infection rate in goats according to geographical distribution

Name of village	No.	Infected No. (%)	*P* value
Sayid Abdil Adheem	4	2 (50)	=0.00001*
Jyzaniyah	3	0	
Besrugiyah	4	0	
Total	11	2 (18.2)	

* Significant correlation with geographical areas

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The cultivation demonstrated the presence of *Brucella*sp in 6 (42.9%) of the RBT positive samples, which were confirmed in RT-PCR and conventional PCRs (Figure 2 and 3). The phylogenetic work revealed close similarity with isolates from India, Egypt, China, and USA (Figure 4).

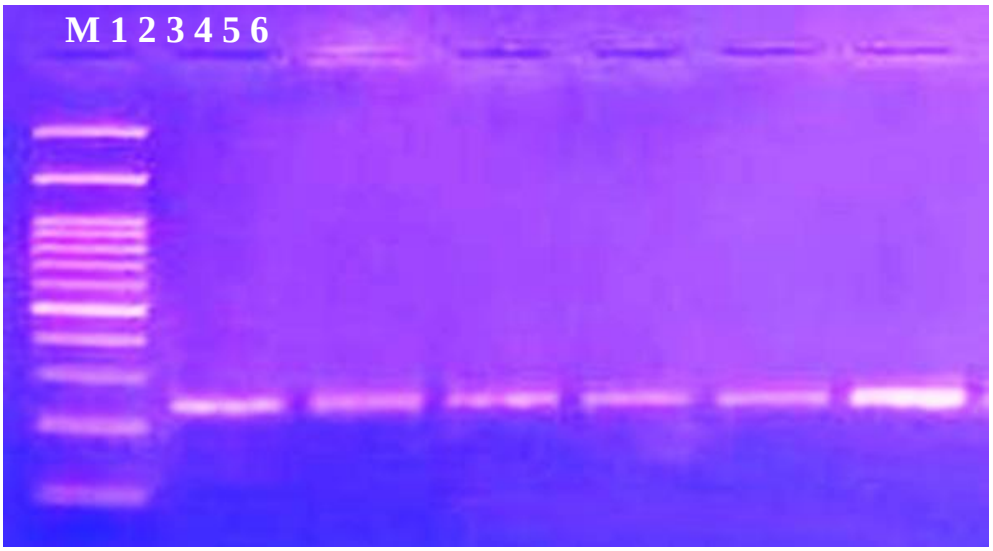

Figure 2: Image of 2%-agarose gel electrophoresis of PCR according to the outer-membrane protein gene region of *Brucella* spp. isolated from blood samples of ruminants. M: ladder. 1-6: Positive PCR products at 223bp.

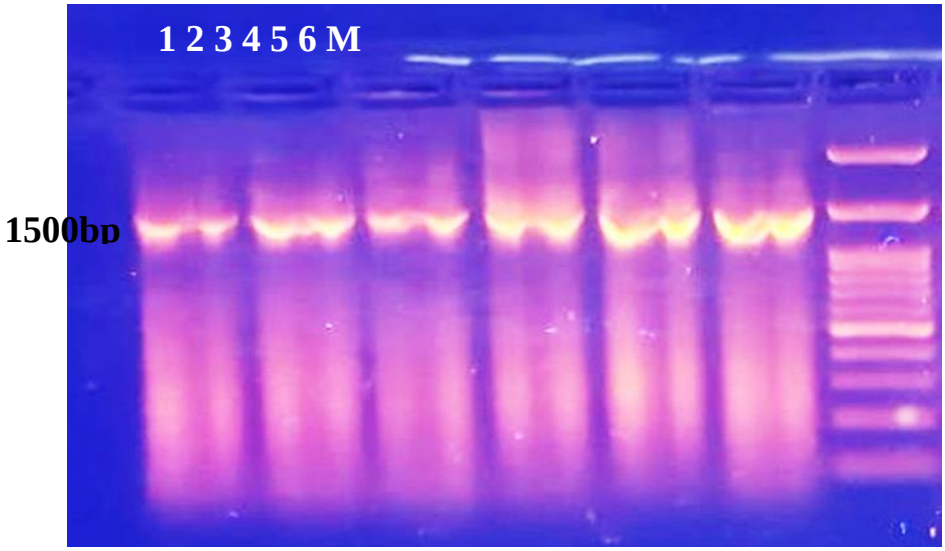

Figure 3: Image of 2%-agarose gel electrophoresis of PCR dependent on the *16S rRNA* gene of *Brucella* spp. M: ladder. 1-6: Positive PCR products at 1500 bp

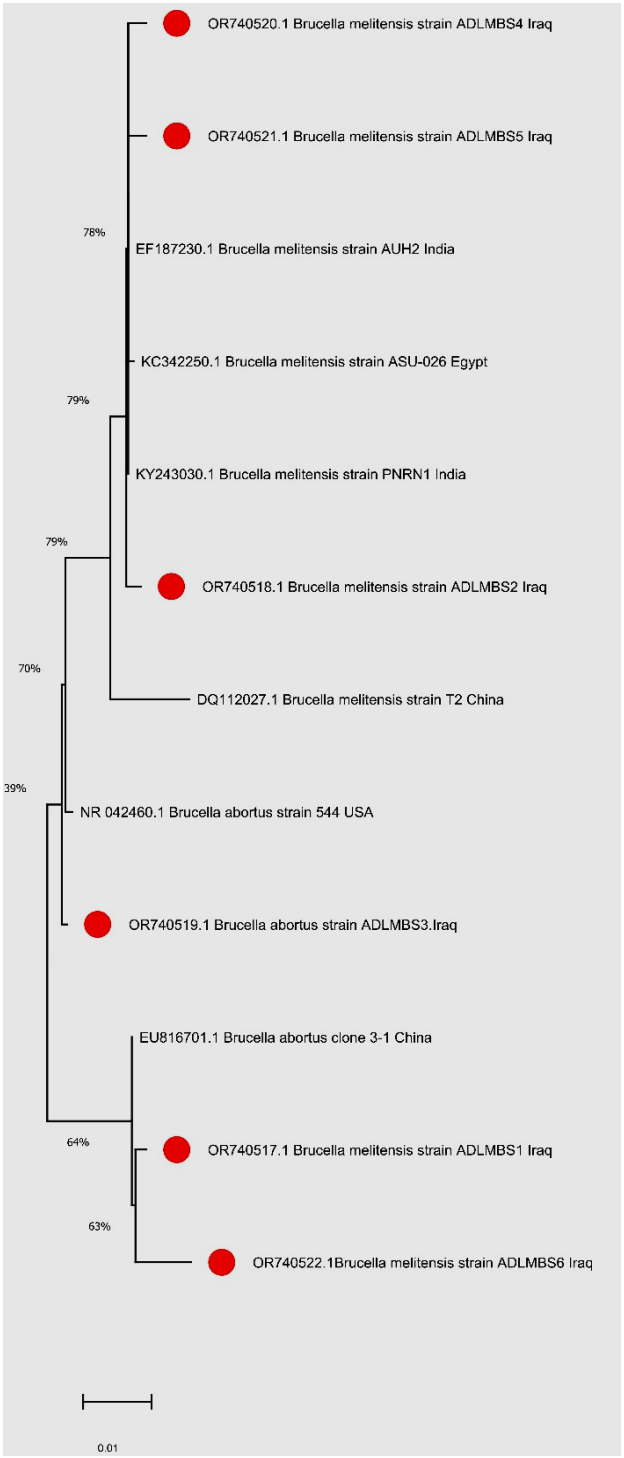

Figure 4-5: *16S rRNA* gene-based phylogenetic tree of *Brucella* spp. isolated from blood samples of ruminants. Red-colored large filled circles: Isolates of present study from Al-Kut City, Iraq

Discussion

Brucellosis is a highly contagious disease that continues to exist in numerous nations and affects both animals and humans. Livestock experts face occupational hazards, while purchasers of animal items are at risk of food-borne illnesses (11). Brucellosis negatively

impacts livestock manufacturing by causing abortions, fertility issues, and a decrease in milk yield. Approximately 95% of the rural community in developing nations relies on livestock as the primary source of their economy. The detection of *Brucella* spp. in diverse farm livestock underscores their contribution to the dissemination of diseases (12–16).

Serological tests cannot distinguish between various species of *Brucella*, as antibodies to the smooth brucellae based lipopolysaccharide (LPS) exhibit a high degree of cross-reactivity. Isolating and identifying the causative pathogen from biological samples is still considered the most reliable approach for epidemiological research and determining resistance to antimicrobial drugs. However, these techniques are both time-consuming and pose potential risks. PCR-based identification of *Brucella* genome in patient specimens is a highly favored method for definitively diagnosing brucellosis. Khan et al (17) found that although the RT-PCR has low sensitivity due to the small quantity of DNA present in the bloodstream, it still obtained the maximum reliability. According to the authors (17), *Brucella abortus* DNA was identified in the blood samples of 13 cows and 18 buffalos. This recent discovery aligns with previous studies that have shown the presence of *B. abortus* in cattle in Pakistan (18-20). Additionally, a study documented the occurrence of *Brucella melitensis* infection in bovines (21).

Utilizing multifactorial model evaluation, Ameen et al (22) identified various risk factors linked to brucella illness in small ruminants through the use of RT-PCR. In overall, the research discovered a notable disparity in Brucella-positive specimens across the districts of Duhok. The results are consistent with a serological investigation conducted in the same region by Alhamada et al (23), which revealed a notably higher prevalence of this pathogen in livestock from three specific regions. However, their findings contradicted a separate serological investigation carried out in the Duhok, which documented lower rates of positive serology in both sheep and goats (24). In Iraq, a separate study by Al-Busultan et al (25) documented an incidence rate of 59.5%, while other studies by Al-Shwany and Robertson (26) and Selim et al (27) showed lower rates. The variations in these findings may be attributed to multiple variables, such as disparities in the agroecological regions examined, the methods and techniques employed for management and production, the number of participants, and the diverse diagnostic procedures utilized (28).

Our results regarding the phylogenetic analysis agree with those by Ameen et al (22), who found that their bacterial sequences were close similar to isolates from countries, such as China. For the close relation with samples from different countries; India, Egypt, China, and USA, importation of animals and human travel could be major reasons for such genetic evolution of the current studied bacterium.

Conclusions

The present study results shows that *Brucella* spp. was present in cows, buffalos, sheep, and goats, which might be considered as important infection sources to spread the infection to different animal herds and areas.

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