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Original Article

Serological and Molecular Detection of Human Brucellosis in Rural Areas in Wasit Province, Iraq

Alqaseer, K[1]*, Al-Khafajy, A. A. M[2], Almkhadhree, E. A. K[2]

1. Department of Basic Science, Faculty of Nursing, University of Kufa, Najaf, Iraq
2. Department of Biology, College of Science, University of Wasit, Wasit, Iraq

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Corresponding Author: kawthera.hasan@uokufa.edu.iq

Abstract

Brucellosis is endemic in Iraq, and annual surveys using advanced diagnostic assays are needed. This study aimed to investigate the prevalence of human brucellosis in rural areas in Wasit province using ELISA and PCR. A total of 276 serum samples were randomly obtained from participants from rural areas in the Wasit province. The results showed that out of 276 serum samples tested by ELISA, 30.07% were positive. Significantly, mild infection was increased compared to moderate, severe and highly severe infections. To confirm the species of *Brucella*, seropositive samples were tested by PCR assay targeting the *BCSP31* gene for *Brucell*a spp. and the *IS711* gene for *B. abortus* and *B. melitensis*. Molecular findings confirmed 30.12% positive samples to *Brucella* spp., including 28% and 44% positives to *B. abortus* and *B. melitensis*, respectively, whereas 28% positive samples to other undifferentiated species of *Brucella*. Association between seropositivity and demographic risk factors, age and gender, were reported to be significantly higher among individuals aged 21-40 (41.91%) and lowered among those aged ≤20 years (13.56%). For gender, a high nominal positivity rate was detected in females (36.07%) than in males (28.37%). Association between the degree of severity of the infection and demographic risk factors recorded that mild infection (75%) was increased among individuals of ≤20 years, while moderate and severe infections were elevated significantly in groups of 21-40 and 41-60 years. The highly severe infections appeared in those aged 21-40 years (15.91%). Regarding gender, mild and moderate infections were elevated significantly in males; whereas severe and highly severe infections were increased significantly in females. In conclusion, this study is the first random epidemiological study investigating the prevalence of human brucellosis in rural areas in Iraq. Undifferentiated species of *Brucella* were detected in PCR-positive results. The incorporation of molecular techniques for the diagnosis will help resolve the *Brucella* genus and detection the primary sources that play roles in the transmission of infection.

Keywords: *Brucella melitensis*, *Brucella abortus*, Enzyme-linked immunosorbent assay (ELISA), Polymerase chain reaction (PCR), Demographic risk factor

1. Introduction

Brucellosis is a common zoonotic infection worldwide, which is caused by a small, non-motile, non-spore-forming, slow growing and Gram-negative coccobacillus belonging to the *Brucellaceae* family in the alpha-2-subclass of the *Proteobacteria* (1). Currently, the genus of *Brucella* has compromised 12 recognized species, of which four-namely, *B. abortus*, *B.canis*, *B. melitensis* and *B. suis*, are the leading causes of the disease in humans (2). The disease has been distributed widely in many countries with high endemicity in numerous areas resulting in severe economic losses in dairy animals and debilitating effects in humans in addition to its complicated

treatment that can turn *Brucella* organisms into candidate agents of biological warfare (3, 4). Person-to-person direct contact and, most significantly, direct and indirect contact with infected-domesticated animals or consumption of their contaminated dairy products are considered the main source of transmission of infection (5). Virulent *Brucella* organisms such as *B. melitensis* can infect both non-phagocytic and phagocytic cells, and the intracellular environment of host cells sustains extensive reproduction, allowing bacterial expansion and subsequent transmission to new host cells (6).

Since the isolation of the organism by David Bruce in 1887 and the zoonotic nature of the disease by Zammit in 1905, features of brucellosis have changed drastically due to many sanitary and socioeconomic factors (7). The symptoms of illness in humans are similar to those of the flu, but brucellosis can affect any organ and body system, presenting non-pathognomonic symptoms that are easily confused with other medical conditions and present various diagnostic difficulties because it mimics many other diseases (8). In addition, there is still no optimal therapy for some particular clinical forms of infection, and there are difficulties with preventive measures in developing countries (9). Conversely, the overdiagnosing of the disease may result in untoward drug effects and, no less importantly, in overlooking other infectious severe or non-infectious diseases (10). In general, the microbiological diagnosis of human brucellosis relies on three different modalities; isolation by culture, serology and molecular polymerase chain reaction (PCR)-based assays (11). For culture detection of *Brucella*, the method is hampered by laboratory safety concerns, reduced sensitivity in prolonged disease and focal infections, and slow-growing features (12).

In contrast, serological techniques as enzyme-linked immunosorbent assay (ELISA), have been developed to be an indirect strategy for probing the patient's immune system in search of antibodies that attest to the previous contact with the pathogen (13). ELISAs often perform as a simple and rapid diagnostic technique with automatic obtaining of results (14), but varying degrees of sensitivity and specificity were reported with different types of ELISA (15). PCR tests have confirmed high sensitivity and specificity for detecting and rapidly diagnosing *Brucella* species in different specimens targeting particular genes (16). During the last two decades, the breakdown of public health systems in resource-poor and politically troubled countries has resulted in new foci of disease in Asia and a worsening situation in many countries, including Iraq (11). Hence, the current study aimed to investigate the prevalence of human brucellosis in some rural areas in Al-Qadisiyah province by indirect ELISA and PCR.

2. Materials and Methods

2.1. Samples

276 individuals from both genders, male and female, in the age range of 8-73 years old, were randomly selected from different rural areas in Wasit province and subjected to venous blood sampling under aseptic conditions using disposable syringes. Each blood sample was divided into two portions; I ml of the samples were placed in the anticoagulant EDTA plastic tube for molecular analysis, whereas 2-4 ml was kept in a free-anticoagulant glass gel tube that centrifuged at 4000 rpm for 5 minutes to obtain the serum samples for serology. All whole blood and Eppendorf tubes of sera were kept frozen until tested.

2.2. Serology

Sera, as well as controls, reagents and coated microplates, were prepared and processed according to the manufacturers' instructions of an indirect ELISA Kit (SunLong Biotech, China) to measure specific antibodies generated against human brucellosis. Absorbance was read at an optical density (OD) of 450 nm using the ELISA Microplate Reader (BioTek, USA). Finally, test effectiveness was determined, whereas the critical value (CUT OFF) was calculated at 0.299, and the samples were considered positive if the OD value was ≥ CUT OFF. Additionally, the seropositive ODs were classified into four categories based on severity of infection mild (≤0.449), moderate (0.450-0.599), severe (0.600-0.799) and highly severe (≥0.800) infections.

2.3. Molecular Assay

Based on Protocol (A) of the G-spinTM Total DNA Extraction Kit (Intron Biotechnology, Korea), DNAs were extracted from the tubes of EDTA-whole blood samples. The purity (A260) and concentration (ng/µl) of all extracted DNA samples were evaluated using the Nanodrop (Thermo-scientific, UK) system at values ranged 1.7-2.1 and 40-138ng/µl, respectively. Three sets of primers were used targeting the BCSP31 gene for *Brucell*a spp.[(B4: (5'-TGG CTC GGT TGC CAA TAT CAA-3′) and B5: (5'- CGC GCT TGC CTT TCA GGT CTG-3′)] and IS711 gene for *B. abortus*[F:(5′-TGC CGA TCA CTT AAG GGC CTT CAT-3′) and R: (5'-GACGAAGAACGGAATTTTTCCAATCCC-3')] and *B. melitensis*[F: (5′-TGC CGA TCA CTT AAG GGC CTT CAT-3') and R: (5′-AAA TCG CCG TCC TTG CTG GTC TGA-3')] as designed by Garshasbi, Ramazani (16). Ready to use AccuPower® PCR PreMix Kit (Bioneer, Korea) was applied to prepare the MasterMix tube at a final volume of 25µl. PCR-reaction was performed in a thermal cycler (BioRad, USA) with a particular condition optimized to each primer obtained in Optimase ProtocolWriter™ available online. Electrophoresis using 1.5% agarose gel stained with Ethidium bromide was performed to detect positive PCR products at 223 bp, 498 bp and 731 bp for *Brucell*a spp., *B. abortus* and *B. melitensis*, respectively.

2.4. Statistical Analysis

All study data were documented, tabled, figured and analyzed statistically using the Microsoft Office Excel 2016 software and GraphPad Prism 6.01 software. Chi-square (x^2) and Odds Ratio were applied to detect significant differences between and among the results of ELISA and PCR at $P<0.05$. Values have been expressed as mean±standard deviation (range) [M±SD (R)] or as number (percentage) [No. (%)].

3. Results

The recorded data showed that out of 276 sera-tested samples by indirect ELISA, 83 (30.07%) samples were seropositive to antibodies against human brucellosis developed against *Brucella*. According to the severity of the infection, the positive samples revealed a significant variation ($P<0.05$) in their values. Significantly, a higher prevalence was observed in mild infections (43.37%) when compared to moderate (27.71%), severe (18.07%) and highly severe (10.84%) infections (Figure 1). Additionally, values of ODs of seropositive samples were 0.391±0.0381 (0.308–0.448), 0.518±0.045 (0.454–0.596), 0.693±0.044 (0.609–0.797), and 1.016±0.213 (0.835–1.448) for mild, moderate, severe and highly severe infections, respectively (Figure 2).

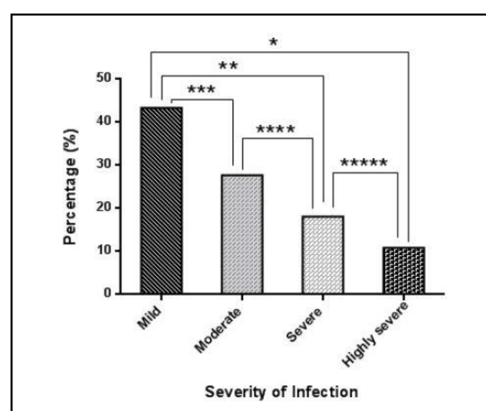

Figure 1. Level of severity of infection among seropositive study individuals
Significant differences were expressed as * ($P≤0.013$), ** ($P≤0.029$), *** (0.036), **** ($P≤0.043$), ***** ($P≤0.048$)

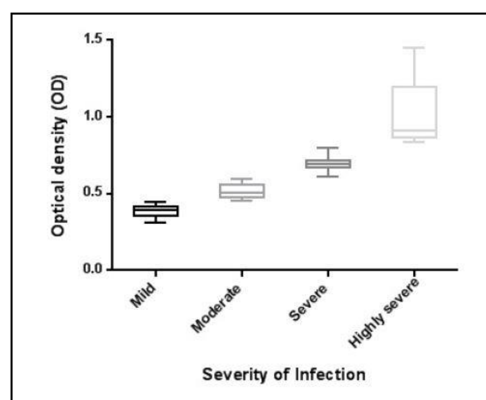

Figure 2. Values of seropositive samples distributed among the levels of infection's severity

To confirm the species implicated in human brucellosis, seropositive samples were tested using the molecular PCR assay targeting the *BCSP31* gene for *Brucell*a spp. and the *IS711* gene for *B. abortus* and *B. melitensis*. The findings of the molecular assay revealed that 25 (30.12%) samples were positive for *Brucella* spp., whereas 7 (28%) and 11 (44%) were positive for *B. abortus* and *B. melitensis*, respectively. However, 7 (28%) positive samples were reported as an undifferentiated species of *Brucella* (Figures 3-7).

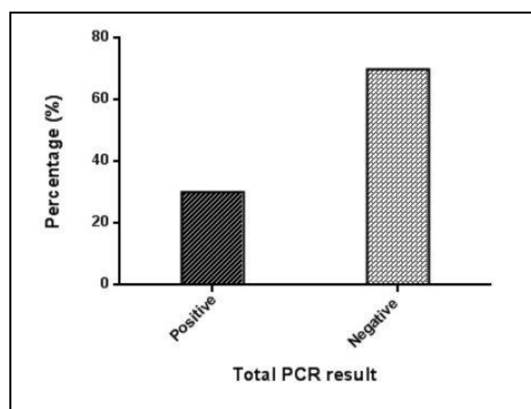

Figure 3. Total positive results for testing 83 seropositive samples by molecular PCR assay

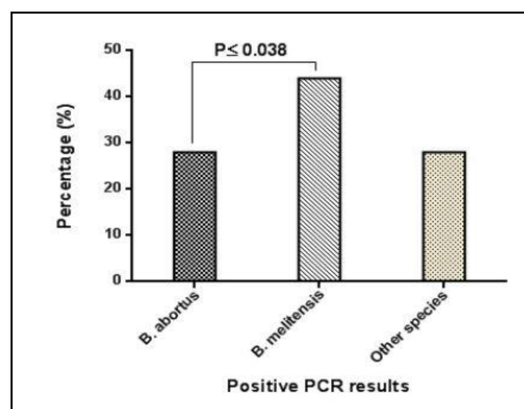

Figure 4. Distribution of 25 positive PCR samples based on the species of *Brucella*

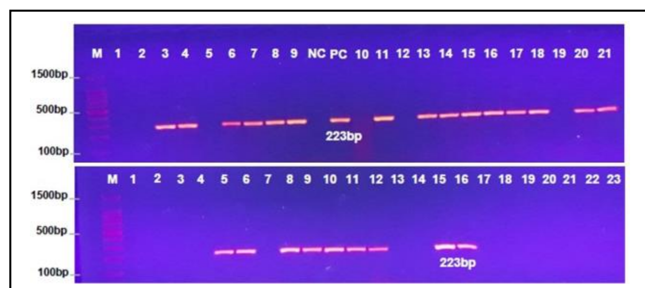

Figure 5. PCR analysis by electrophoresis using 1.5% agarose gel stained with Ethidium bromide at 80 Volt and 100 Am for 1.5 hours, targeting BCSP31 gene of *Brucella* spp.
Lane (M) represents the ladder marker (100-1500bp).
Lane (NC) represents negative control.
Lane (PC) represents positive control.
Lanes (1, 2, 5, 10, 12, and 19 of 1st image; and 1, 2, 3, 4, 7, 13, 14, 17, 18, 19, 20, 21, 22, and 23 of 2nd image) represent negative samples for human brucellosis.
Lanes (3, 4, 6, 7, 8, 9, 11, 13, 14, 15, 16, 17, 18, 20 and 21 of 1st image; and 5, 6, 8, 9, 10, 11, 12, 15 and 16 of 2nd image) represent positive samples for human brucellosis (*Brucella* spp.) at 223bp

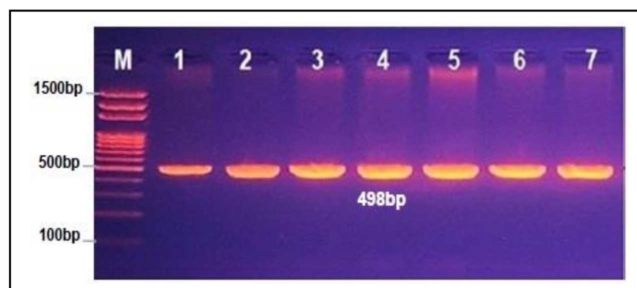

Figure 6. PCR analysis by electrophoresis using 1.5% agarose gel stained with Ethidium bromide at 80 Volt and 100 Am for 1.5 hours, targeting the IS711 gene of *B. abortus*.
Lane (M) represents the ladder marker (100-1500bp).
Lanes (1-7) represent positive samples for human brucellosis (*B. abortus*) at 498 bp

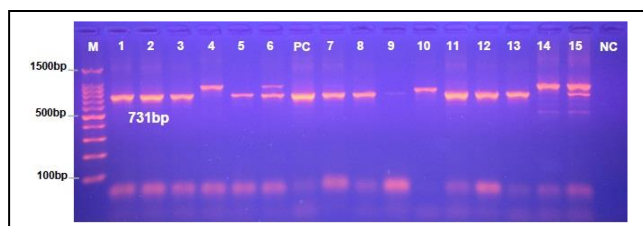

Figure 7. PCR analysis by electrophoresis using 1.5% agarose gel stained with Ethidium bromide at 80 Volt and 100 Am for 1.5 hours, targeting the IS711 gene of *B.melitensis*.
Lane (M) represents the ladder marker (100-1500bp).
Lane (NC) represents negative control.
Lane (PC) represents positive control.
Lanes (1, 2, 3, 5, 6, 7, 8, 11, 12, 13, and 15) represent positive samples for human brucellosis (*B. melitensis*) at 731bp.
Lanes (4 and 14) consider a representative image for other differentiated human *Brucella* species.
Lane (9) considers a representative image for the negative samples of human brucellosis

The Association of seropositive findings to different groups of age and gender reported a significant variation in their values (Table 1). For age, significantly higher values were reported individuals aged 21-40 years (41.91%) followed by those aged 41-60 years (30.12%) and > 60 years (20.69%), and the lowered value was detected among study individuals aged ≤20 years (13.56%). Additionally, the group of 41-40 years appeared to be at higher risk of infection with brucellosis (2.44) than other age groups; 1, 0.576 and 0.297 for 41-60, >60 and ≤20 years, respectively.

For gender, although females showed a higher rate of positivity (36.07%) than males (28.37%), no significant differences (P>0.05) appeared between females and males. However, females were exposed significantly to a double risk of infection (1.424) than males (0.702).

The Association between the severity of infection of seropositive individuals and demographic risk factors showed significant differences (P<0.05) in their values (Table 2). In comparison between age groups, seropositive individuals aged ≤20 years showed a significant increase in mild infection (75%) when compared to other age groups;21-40 (34.09%), 41-60 (44%) and >60 years (66.67%). The moderate and severe infections were elevated significantly in groups of 21-40 years (27.27% and 22.73%, respectively) and 41-60 years (28% and 20%, respectively) more than in groups of ≤20 (16.67% and 6.67%, respectively) and >60 (16.67% and 16.67%, respectively) years. However, highly severe infections increased significantly in study individuals aged 21-40 years (15.91%) and decreased significantly in study individuals of 41-60 years (8%). However, severe infection was absent completely in age groups of ≤20 and >60 years. Additional comparison between degrees of infection revealed a significant elevation in values of mild infection at all age groups; ≤20 years (75%), 21-40 years (34.09%), 41-60 years (44%) and >60 years (66.67%).

Regarding gender, mild and moderate infections showed a significant elevation in males (52.46% and 32.79%, respectively) when compared to females (18.18% and 13.64%), whereas severe and highly severe infections were increased significantly in females (40.91% and 27.28%, respectively) in comparison with males (9.84% and 4.92%, respectively). An additional comparison between degrees of infection revealed a significant elevation in values of males with mild infection (52.46%) and females with severe infection (40.91%).

Table 1. Association of seropositive by ELISA to demographic risk factors, age and gender

Factor	Total No.	Positive	Odd ratio	Risk
Age (Year)				
≤ 20	59	8 (13.56%)	0.297	0.39
21-40	105	44 (41.91%) *	2.44 *	1.838 *
41-60	83	25 (30.12%)	1	1
>60	29	6 (20.69%)	0.576	0.664
P-value	-	0.025	0.038	0.033
Gender				
Female	61	22 (36.07%)	1.424	1.271
Male	215	61 (28.37%)	0.702	0.787
P-value	-	0.078	0.039	0.042

Significance * ($P<0.05$)

Table 2. Association between degrees of infection of seropositive individuals and demographic risk factors, age and gender

Factor	Total No.	Mild	Moderate	Severe	Highly severe	*P*-value
Age (Year)						
≤ 20	8	6 (75%) *	1 (16.67%)	1 (6.67%)	0 (0%)	*0.007*
21-40	44	15 (34.09%)	12 (27.27%) *	10 (22.73%) *	7 (15.91%) *	*0.045*
41-60	25	11 (44%)	7 (28%) *	5 (20%) *	2 (8%)	*0.048*
>60	6	4 (66.67%)	1 (16.67%)	1 (16.67%)	0 (0%)	*0.018*
P-value	-	0.011	0.039	0.021	0.028	-
Gender						
Female	22	4 (18.18%)	3 (13.64%)	9 (40.91%) *	6 (27.28%) *	*0.026*
Male	61	32 (52.46%) *	20 (32.79%) *	6 (9.84%)	3 (4.92%)	*0.019*
P-value	-	0.029	0.025	0.036	0.041	-

Significance * ($P<0.05$) between vertical values

4. Discussion

Human brucellosis is a neglected zoonotic problem worldwide with a high degree of morbidity in humans and is mostly overlooked due to other febrile conditions. Also, it is a pervasive disease of animals having potential transmission through direct contact with infected animals and/or their discharges, as well as consuming their products such as milk, milk products and meat (14). Our findings revealed that the total seropositivity of human brucellosis was 30.07%. In comparison with other studies, the seroprevalence of human brucellosis was 14.96% in Ethiopia (17), 13.13% in Pakistan (18), 17% in Uganda (19), 18% in Turkey (20), 23.3% in Sudan (21), and 29.5% in Iran (22). In Iraqi rural areas, the factors strongly associated with human brucellosis involved frequent consuming of raw milk and their products, keeping animals at or near a home, direct contact with discharges, particularly during parturition, and slaughtering of animals.

Based on data from seropositive samples, we showed that mild titer of positive infections was more prevalent than moderate, severe and highly severe infections. These findings may indicate the positive individuals were either exposed previously or very recently to brucellosis (11, 23). Serological techniques such as ELISA demonstrated a relatively high rate of sensitivities and specificities in the detection of brucellosis; however, asymptomatic and self-limiting episodes of *Brucella* infection are not uncommon in regions where the disease is endemic, and IgG isotype antibodies may persist for many months after the conclusion of successful antibiotic therapy (24, 25).

This explains the high seroprevalence of anti-*Brucella* antibodies found in areas of endemicity and among individuals repeatedly exposed to the organism (26, 27). In contrast to other pathogenic bacteria, no classical virulence factors have been described in *Brucella* organisms; instead of this, some molecular determinants were discovered as virulence elements that allow the bacterium to invade, resist intracellular killing and reach their reproductive niche in professional or non-professional phagocytes (6, 28). Baldi, Miguel (29) found that some patients had low or negative titers of antibodies despite persistent brucellosis, suggesting that this might be due to blocking or incomplete IgG antibodies. Many studies suggested that humoral immunity's role against intracellular bacterial infections may be limited and not protective.

Additionally, antibody-mediated opsonization by immunoglobulins (IgM, $IGg_{1,\ 2a,\ 3}$, and IgA) could enhance the phagocytic uptake of bacteria and limit the level of initial infection with *Brucella* but with little effect on the intracellular course of infection (30, 31). Nelson and Solotorovsky (32) recorded that though the humoral antibodies play some role in resistance to the organism, cell-mediated immunity appears to be the principal recovery mechanism. Alsubaie, Turkistani (33) mentioned that the high titer of antibodies indicated a possibility of relapse or progression to chronic focal disease and concluded that *Brucella* serology does not correlate with clinical outcomes or culture positivity.

Although several serological assays are available in clinics, numerous researchers mentioned that none of them meets the standard criteria for a convincing diagnosis, none of them is recommended to be used alone in endemic areas, and a verification test is often required (27, 34). In recent years, PCR-based techniques are promising alternatives for diagnosing brucellosis as they have proved to be faster and more sensitive than other diagnostic methods (35). In this study, the application of molecular PCR assay on seropositive samples revealed 30.12% positive samples to the genus of *Brucella*, which included 28% positives to *B. abortus*, 44% positives to *B. melitensis*, and 28% undifferentiated species of *Brucella*. Our findings were consistent with the view that *B. melitensis* is the most significant human pathogen in *Brucella* species globally (36, 37). In Greece, Mitka, Anetakis (38) confirmed that the percentage of PCR-positive results among 200 acute brucellosis patients was 99%, while in Turkey, Surucuoglu, Ural (39) revealed that 88% of patients had positive PCR results. In Saudi Arabia, Asaad, Alqahtani (40) reported that PCR correctly diagnosed 59.26% and 11.18% of acute and chronic brucellosis patients, respectively. In this study, 69.88% of seropositive samples were negatived by PCR. This high seropositivity rate might be a reflex for past exposure to the organism and cross-reaction or PCR-inability to detect the bacterium due to the low bacterial load in the blood of patients with chronic brucellosis and inhibitory effects taking place from surrounding substances (24, 40). Other factors include lack of optimal clinical specimen and storage conditions, with low sample volume, in addition to the loss of standardization and uniformity concerned PCR protocol such as inappropriate experimental design, target gene and primer, extraction method and PCR amplification (38-40). However, PCR-based detection of *Brucella* remains a novel and much more efficient diagnostic tool not only to detect but also to accurately distinguish between subacute, acute and chronic infection (27). For undifferentiated *Brucella* species detected in the current study, different reports referred that other members of *Brucella* species, such as *B. suis* (41), *B. canis* (42), *B. ovis* (43), and *B. neotomae* (44), could be important in human brucellosis.

Our findings showed a significant variable distribution among the demographic risk factors for seropositive findings. For age, the prevalence of human brucellosis was more common in individuals aged 21-40 years. Although we detected that mild infection is elevated significantly at all age groups, it was more

significant at 20 years old, whereas moderate and severe infections were increased at 21-40 years and 41-60 years but not for a highly severe infection that appeared significantly at 21-40 years. In Iraq, Daood, Zajmi (45) recorded a higher seroprevalence (34.3%) among the age group of 31-40 years. Tay, Ahmad (46) showed that human brucellosis is prevalent in Malaysia patients ranging from 20-45 years old. Guler, Kokoglu (47) concluded that the mean age of the enrolled patients in Turkey was 39.6±18.2 years. In an epidemiological study carried out in Saudi Arabia, the findings observed that most cases of the patients occurred between 20-30 (21%) and 31-40 (17%) years (48). Mancini, Bella (49) summarized that no significant differences were observed between age groups in Italy.

For gender, females showed an insignificant higher prevalence of human brucellosis than males; however, mild and to less degree moderate infections appeared more prevalent in males, while severe and to less degree, highly severe infections were more pronounced in females. Our findings agreed with other researchers' observations (47, 50). In a recent Iraqi study involving 385 patients (45), the results showed that females had higher seropositivity than males in different years; 2017 (38.9% and 23.1%, respectively), 2018 (34.6% and 26%, respectively) and 2019 (16.2% and 11.2%, respectively). In contrast, other studies reported that the disease is more common in males than females (51, 52). We suggested that a slight increase in the prevalence of the disease among females might be attributed to the fact that; females are commonly involved in handling livestock and their products. In most rural areas, milking is still done by hand, and the largest share of milk is used to produce typical cheeses for the local markets or directly sold by farmers to consumers.

5. Conclusion

Surprisingly, no random epidemiological studies were performed previously or recently to detect the prevalence of human brucellosis, particularly in rural areas in Iraq. This makes our work the first serological and molecular study performed using advanced diagnostic assays, indirect ELISA and PCR. Our findings confirmed that *B. melitensis* was the more prevalent implicated cause of human brucellosis besides *B. abortus*; however, undifferentiated species of *Brucella* were found among positive individuals. Therefore, the incorporation of molecular techniques for the diagnosis will help resolve the *Brucella* genus and the main sources that play roles in the transmission of infection.

Authors' Contribution

Study concept and design: K. A. and A. A. M. A.
Acquisition of data: E. A. K. A.
Analysis and interpretation of data: K. A.
Drafting of the manuscript: A. A. M. A.
Critical revision of the manuscript for important intellectual content: K. A.
Statistical analysis: E. A. K. A.
Administrative, technical, and material support: A. A. M. A.

Ethics

The current study's design was licensed by the Scientific Committees in the College of Science, University of Wasit (Wasit, Iraq) and the Faculty of Nursing, University of Kufa (Najaf, Iraq).

Conflict of Interest

The authors declare that they have no conflict of interest.

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