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Serological and Molecular Detection of *Brucella Melitensis*, Wasit, Iraq

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Abstract
The current study was carried out to identify the prevalence of *Brucella melitensis* infections in humans in Wasit province serologically using the Rose-Bengal test (RBT) and molecularly by the polymerase chain reaction (PCR). The venous blood samples collected randomly from totally 300 individuals into anticoagulant-EDTA plastic tube were tested initially by RBT, and the positive samples were confirmed later by the PCR assay. The findings revealed that 24.67% (74/300) of study population was serologically positives by RBT. Molecularly, there were 39.19% (29/74) positive individuals by the conventional PCR. Regarding the region, significant increases (P<0.0456) in positivity were reported in Al-Numaniyah (17.33%) while significant reduction was seen in Badra (8%) when compared to other study regions; Al-Kut (8%) and Al-Hai (10.67%). In relation to the study period, insignificant differences (P<0.0581) were detected between the positive values of August (8%) and September (11.33%). For sex, the positive higher values were identified significantly (P<0.0363) in males (12.21%) than females (3.45%). Among age groups, the findings of positivity were increased significantly (P<0.04) in >20-40 years old (9.8%) and > 40 years old (10.92%) than those of ≤20 years old (3.57%). In conclusion, the combination of different diagnostic methods provided more insurance and supported high reliably findings. Also, the application of molecular-PCR assays is of great value since it provides a more sensitive and specific data about the status of infection in addition to their ability in detection of organism in blood samples efficiently. In people and animals living in the same households, brucellosis allows for identification and quantification of risk factors for brucellosis transmission at the household level. However, the prevalence of infection in animal reservoirs can provide a key to its prevalence in humans; therefore, surveys of brucellosis in different domestic and wild animals are required
Keywords: Brucellosis, Rose-Bengal, PCR, Season, Age, Sex

Introduction

Brucellosis as one of the ancient and most widespread zoonotic diseases affects humans as well as the animals, a bacterial disease resulted by a Gram-negative, facultative intracellular bacterium of the genus *Brucella* (Pradeepkiran et al., 2021). Currently, the scientifically communities referred for 6 separately species that having different morphological characteristics based on presence or absence the *O*15 polysaccharide (OPS) in its smooth lipopolysaccharide (SLPS) that covering the outer membranes (Coelho et al., 2019; Akoko et al., 2021). Many researchers thought that SLPS have the ability for evading innate immunity as well as for inducing the inflammation by activation the cytokines. This evasion technique increase the virulence of SLPS when compared to rough lipopolysaccharide (RLPS) strain,

unable for inhibiting the immune responses of the host and greatly impact the innate immunes (Dykman, 2020; Shatti et al., 2021). Other differences are the cleavages of DNAs through a phage specifically for *Brucella* as approximately 40 phages could causes a fully lysing in the genomes of one or more species. R/C phages cause lysing of phages in RLPS of Tb, Wb, and Iz1 differentiating of species within SLPS are susceptibly lysing with the Iz1 and have different lysing with WB (Matope et al., 2009; Tevdoradze et al., 2015; Saxena, 2021). Additionally, variations are not found between the species of *Brucella*, but exist in other types of *Brucella* that having several distinct biovars. This difference in biovar has variation in biochemically and growing feature like variation in capabilities for growing within the existence CO_2, to result in H_2S, to agglutinating the A and M antisera, and to growing with existence of urea (Al-Mariri, 2015; García Lobo et al., 2019).

In a number of developed countries, brucellosis has been eradicated through test and slaughter, vaccination and restriction of animal movements (Bahmani and Bahmani, 2022). However, many developing countries remain with the highest disease incidence rates such as those located within Asia (Laine et al., 2022). In Iraq, human brucellosis was confirmed firstly by Al-Zahawi in 1937 (Hussein et al., 2019); however, recent information about the annual prevalence of brucellosis remained low and need to furthermore support. Therefore, the current study was carried out to identify the prevalence of *B. melitensis* infections in humans in Wasit province (Iraq), serologically using the RBT and molecularly by the conventional PCR.

Materials and methods

Ethical approval

The study approves by the Scientific Committee of the Department of Basic Science in the College of Density (University of Wasit, Wasit, Iraq).

Samples

Totally, 300 individuals of different ages and sexes were selected randomly from different areas in Wasit province (Iraq) during August and September (2023). Each individual was subjected for draining 5 ml of venous blood into glass vacutainer, and then divided equally into an EDTA-anticoagulant plastic tube and free-anticoagulant glass gel tube. The first tube was kept frozen for later molecular testing while the second tube was centrifuged (5000 rpm / 5 minutes) and the obtained sera were tested directly by the RBT.

RBT

As described by Al-Hassani et al. (2018), the Rose-Bengal Antigen Kit (Wuhan Ecalbio, China) was served to detect the suspected seropositive samples to brucellosis.

Conventional PCR

According the manufacturer instructions of the G-spin Total DNA Extraction Kit (Intron, Korea), the EDTA-blood samples were thawed in water bath and processed for extraction of DNAs. All extracted DNAs were tested spectrophotometrically using the Nanodrop System (Thermo-scientific, UK) to detect the concentration and purity of each sample. Targeting the *16S rRNA* gene, one set of primers was designed [(F: 5´-AGG CCC TAG GGT TGT AAA GC-´3) and (R: 5´-GTT TAC GGC GTG GAC TAC CA-´3)] based on the GenBank-NCBI data (OR053959.1), the MasterMix tubes were prepared at a final volume of 20 µl. PCR reaction was carried out using the thermocycler system as following; 1 cycle initial denaturation (95°C/5 Minutes); 35 cycles of denaturation (95°C/30 seconds), annealing (58°C/30 seconds) and extension (72°C/30 seconds); and 1 cycle final extension (72°C/7 minutes). Electrophoresis

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using the agarose-gel (1.5%) stained with Ethidium Bromide was performed at 80AM and 100 Volt for 90 minutes. According to bands of the standard Ladder Marker (100-1500 bp), the positive PCR products were identified by the ultraviolet (UV) transilluminator (Clinex, China) at approximately 378 bp.

Statistical analysis

The *t*-test in the GraphPad Prism Software (*version 6.0.1*) was applied to estimate significant differences between the obtained results at * (P<0.05), (Ibraheim et al., 2023).

Results

The findings revealed that 24.67% (74/300) of study population was serologically positives by RBT (Figure 1). Molecularly, there were 39.19% (29/74) positive individuals by the conventional PCR (Figures 2, 3)

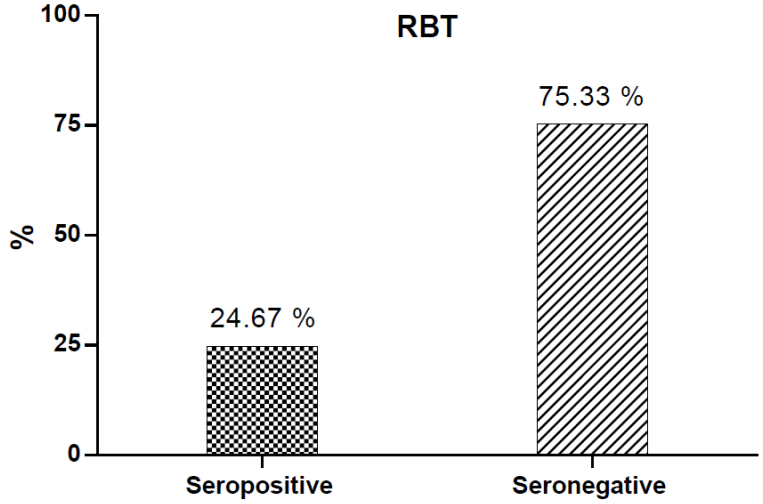
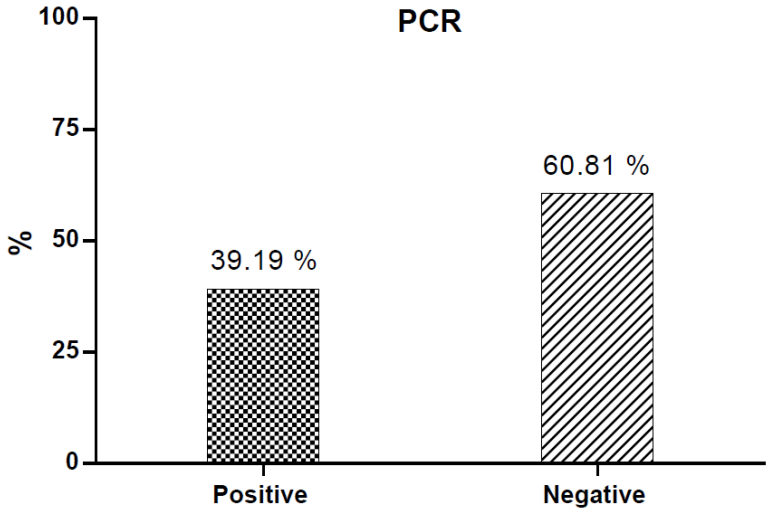


Figure (1): Total results of RBT among totally 300 serum samples



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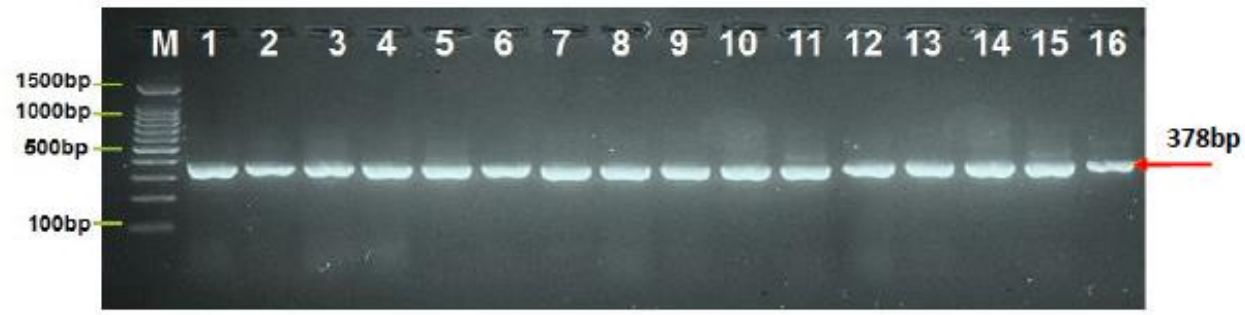

Figure (3): Agarose-gel electrophoresis of some positive PCR products at 80Am and 100 Volt for 90 minutes; Lane M represent ladder marker (100-1500 bp), while lanes 1-16 represent positive samples at 378 bp

Regarding the region, significant increases (P<0.0456) in positivity were reported in Al-Numaniyah (17.33%) while significant reduction was seen in Badra (8%) when compared to other study regions; Al-Kut (8%) and Al-Hai (10.67%), (Table 1).

Table (1): Association of positive PCR results to region factor (Total No: 29)

Factor	**Group**	**Total No.**	**Positive**	
Region	Al-Numaniyah	75	13	17.33 *
	Al-Kut	75	6	8
	Badra	75	2	2.67
	Al-Hai	75	8	10.67
	P-value			0.0456

In relation to the study period, insignificant differences (P<0.0581) were detected between the positive values of August (8%) and September (11.33%), (Table 2).

Table (2): Association of positive PCR results to period factor (Total No: 29)

	Group	**Total No.**	**Positive**	
Period	August	150	12	8
	September	150	17	11.33
	P-value			0.0581

For sex, the positive higher values were identified significantly (P<0.0363) in males (12.21%) than females (3.45%), (Table 3)

Table (3): Association of positive PCR results to sex factor (Total No: 29)

Factor	**Group**	**Total No.**	**Positive (No: 29)**	
Sex	Female	87	3	3.45
	Male	213	26	12.21 *
	P-value			0.0363

Among age groups, the findings of positivity were increased significantly (P<0.04) in >20-40 years old (9.8%) and > 40 years old (10.92%) than those of ≤20 years old (3.57%), (Table 4).

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Table (4): Association of positive PCR results to age factor (Total No: 29)

Factor	Group	Total No.	Positive (No: 29)	
Age	≤ 20	28	1	3.57
	> 20-40	153	15	9.8 *
	> 40	119	13	10.92 *
P-value				0.04

Discussion

In Iraq, the disease remains one of the major disease problems affecting the livestock and human health. In comparison with our findings, the overall prevalence of brucellosis was 23% in Iraq (Shatti et al., 2021), 8.8% in Kyrgyzstan (Bonfoh et al., 2012), 26.7% in Turkey (Yumuk and O'Callaghan, 2012), 16.4% in Kenya (Osoro et al., 2015), 17% in Uganda (Tumwine et al., 2015), 7.2% in Portugal (Pelerito et al., 2017), 9.44% in Egypt (Abdelbaset et al., 2018), 12.8% in Saudi Arabia (Alkahtani et al., 2020) and 34.88% in Mexico (Guzmán-Bracho et al., 2020). This difference in prevalence of *B. melitensis* might follow diagnostic assay used in investigation (serological type, sensitivity and specificity of the instruments used, specimen type, target antigen or antibody), target population (age, breed, individual or group, herd size, sampling and data collection methods) and environment (geographical location of the study area, presence of other domestic and/or wild animals, treatment, prevention and control). Duran-Ferrer et al. (2004) showed that high levels of circulating antibodies are associated with active *Brucella* infection. Several studies have shown that antibody persists for years, even after successful treatment and complete recovery from acute brucellosis, in healthy animals exposed to repeated exposure to organism (Buchanan et al., 2004; Gupta et al., 2010; Almuneef and Memish, 2013).

As serological assays affected by cross-reactivity, low sensitivity and specificity in diagnosis of *Brucella* species, it is recommended to using a more practical, faster, safer, simpler, and accurate technique as molecular PCR that shown to be more sensitive in diagnosis of brucellosis in pure cultures (Sergueev et al., 2017), blood (Açıkgöz et al., 2018), milk and cheese (Altun et al., 2017) and naturally infected animal organs (Akhtar et al., 2017). PCR-based methods are highly applicable and effective for diagnosing acute brucellosis in early stages of disease, studying predictive biomarkers for post-treatment management, and monitoring disease progression for early detection of recurrence (Al-Hassani et al., 2018; Dadar et al., 2019). The PCR method has several advantages in the diagnosis of human brucellosis, including rapidity, safety, high sensitivity, and specificity. Also, it can be considered to complement the traditional methods adopted by serology and/or culture (Rahimi et al., 2020; Kılıç et al., 2021). Similarly, detection of *Brucella* by PCR has also emerged as a new, more effective diagnostic tool for different stages of diseases (acute, subacute and chronic) and different clinical specimens such as serum, urine, cerebrospinal fluid, synovial or pleural fluid, and pus (Ulu Kilic et al., 2013; Sulayman et al., 2020; Di Bonaventura et al., 2021).

Changes in the prevalence of brucellosis in the areas studied are consistent with changes reported by other researchers (Al-Mossawy, 2017; Al-Hakami et al., 2019; Shatti et al., 2021). Ingestion, direct contact, and inhalation are the main routes of infection, but the relative

importance of the method and route of transmission of the pathogen varies depending on the epidemiological region, pet store, professional group, and exposed consumer (Mufinda et al., 2017; Al-Zubaidy, 2018; Gharban et al., 2023). Buzgan et al. (2010) conducted a retrospective analysis of 1,028 incidents occurred between 1998 and 2007, with explaining that these cases might be linked to consumption of unpasteurized dairy products or contact with animals, reflecting the rural nature of the area. Socioeconomic and educational factors are also independent risk factors as concluded by Araj and Azzam (1996) as the risk of brucellosis is higher among people in high-risk occupations. In contrast, Sofian et al. (2008) showed that occupation and education level are not significant risk factors. However, infection in humans may be related to various factors such as lifestyle, economic level of social life, social eating habits and animal management (Alavi et al., 2007; Bosilkovski et al., 2015). A number of studies recorded that handling of infected animals, caring of newborns, cleaning animal nests, and disposing to aborted fetuses increased the risk of infection in certain areas than other (Shareef, 2006; Al-Mossawy, 2017).

The association of brucellosis with temporal or seasonal factors is controversial because some symptoms could persist for long time. Additionally, the incubation period of brucellosis is usually 1–3 weeks, but it may take several months for symptoms of infection to appear (Seleem et al., 2010; Jaffe, 2016). However, seasonal distribution of human brucellosis may indicate that the weather of this month is most favorable to survival of *Brucella* and spreading of infection (Zhou et al., 2018). Alkahtani et al. (2020) showed that the seasonal nature of brucellosis may be associated with various risk factors including rain, sunlight, and consumption the products of infected animals in large numbers. Pal et al. (2020) revealed that brucellosis can occur at any time of a year, but the peak incidence of human brucellosis might closely relate to the month of birth and gestation in field animals due to increasing the excretion of *Brucella* at birth into the environment.

There is debate about the relationship between the prevalence of brucellosis and the sex factor. Although, various studies shown that females are more affected by brucellosis than males (Daood et al., 2020; Ibrahim et al., 2021; Shatti et al., 2021), others reported no gender association to susceptibility (Ahmed et al., 2010; Rahman et al., 2012; Al-Mossawy, 2017). In agreement with our findings, studies conducted in Uganda (Tumwine et al., 2015) and Saudi Arabia (Alkahtani et al., 2020) showed the gender-specific results and a high prevalence of disease in males. Jabary and Al-Samarraee (2015) observed that sexually mature animals are more motivated than immature animals, which might be related to the fact that sex hormones, meso-erythritol present in males (testicles and seminal glands) and erythritol found in allantoic fluid of females stimulate the growing and reproduction of *Brucella*.

Statistical analysis of the study data showed the role of age as a risk factor to brucellosis. In Iraq, Daood et al. (2020) found that the prevalence of brucellosis was highest in males in the age group (31–40 years), followed by >40 and 11–20 years old. Buzgan et al. (2010) found that the prevalence of brucellosis was highest in patients aged 14 to 34 years, which might be reflected by obtaining immunity against secondary infections as the population ages. A study conducted in Lebanon found that the incidence of brucellosis increases with age (Kalaajieh, 2000). Tumwine et al. (2015) showed that young people in school and middle-aged people engaged in professional activities are more susceptible to brucellosis. Another study found that brucellosis is more common in young people especially where raising livestock existed at an

early age (Gur et al. 2003). Alkahtani et al. (2020) observed the high incidence of brucellosis in adults due to the greater frequency of exposure of older ages to infected animals, and children at low risk because the less likely come into contact with infected animals. Rahman et al. (2012) showed that people aged 40 to 80 years are more susceptible to brucellosis. However, Sofian et al. (2008) did not show any significant difference in terms of age factors.

Conclusion

The combination of different diagnostic methods provided more insurance and supported high reliably findings. Also, the application of molecular-PCR assays is of great value since it provides a more sensitive and specific data about the status of infection in addition to their ability in detection of organism in blood samples efficiently. In people and animals living in the same households, brucellosis allows for identification and quantification of risk factors for brucellosis transmission at the household level. However, the prevalence of infection in animal reservoirs can provide a key to its prevalence in humans; therefore, surveys of brucellosis in different domestic and wild animals are required.

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Conflicts of interest

No.

Funds

No external funds were received.

Data availability

All data were used within this manuscript.

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