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Original Article

Expression of *IL1β* Gene in the Placentas from Humans and Cows during *Brucella* Infection

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Abstract

Brucellosis is an important contagious disease affecting most domestic and mature animals. Since the impact of *IL-1β* in *B. abortus* invasion and survival remains elusive, the current study sought to elucidate the actual roles of these potent cytokines in the modulation of the initial immune response to *Brucella* infection. Therefore, this study aimed to detect *Brucella abortus* in the placenta of aborted women and cows and estimate the expression of the interleukin 1β (*IL1β*) gene associated with immune response mechanisms to *Brucella abortus* infection. The detection of *Brucella abortus* was performed by Rose Bengal Test (RBT) and Polymerase Chain Reaction based *AlkB gene (AlkB*-PCR) in the sera and placenta samples of aborted women and cows, respectively. The overall percentage of *Brucella abortus* infection was 13.1% and 5% as determined by RBT and *AlkB*-PCR in aborted women's sera and placentas, respectively. On the other hand, the overall percentage rates of *Brucella abortus* infection in the sera and placentas from aborted cows were 30% and 11% as estimated by RBT and *AlkB*-PCR, respectively. The results of RBT demonstrated that the association between *Brucella abortus* and abortion in cows was statistically significant. On the other hand, it was found that the association between *Brucella abortus* and abortion in women was not significant. Moreover, according to the results of *AlkB*-based *PCR*, the association between *Brucella abortus* and abortion was statistically significant in aborted cows, while it was not significant in aborted women. The sensitivity, specificity, and accuracy of RBT were calculated as 60.00, 53.85, and 54.55%, respectively. Moreover, positive and negative predictive values were reported as 14.33% and 91.28%, respectively. Regarding RBT for aborted cows, the sensitivity, specificity, and accuracy of the test were 81.82%, 57.78%, and 62.49%, respectively. The positive predictive value was reported as 32.08%, while the negative predictive value was reported as 92.88%. Quantitative PCR (qPCR) was carried out for the evaluation of Interleukin 1 Beta (*IL1β*) gene expression. The qPCR result was presented as a fold change in gene expression. A significant increment of *IL1β* gene expression was observed in aborted women (114.905±99.661) and cows (22.454 ±18.528), compared to non-aborted women (4.953±5.564) and cows (2.033±1.845). Statistical comparison of IL*1β* gene expression between aborted women and cows illustrated a non-significant increment in *IL1β* gene expression in aborted women (114.905±99.661), compared to aborted cows (22.454 ±18.528).

Keywords: Abortion, *ALK-B* gene, *Brucella abortus*, Gene expression *IL1β*

1. Introduction

Brucellosis is an important contagious disease affecting most domestic and mature animals. Nonetheless, the exact organs which are mostly infected are the reproductive and sexual organs of both genders. *Brucella* species are intracellular gram-negative bacteria that cause the disease in both animals and humans Smith (1). It has been demonstrated that the bacterial virulence action depends on its ability to prevent *Brucella* phagosome maturation by a special

mechanism that has not been fully understood yet. Therefore, it could result in good multiplications via phagocyting activities of the macrophages and the epithelial cells; moreover, the trophoblasts of the placental can cause a chronic infection (2).

González-Espinoza, Arce-Gorvel (3) reported that *Brucella* could disseminate to other organs, such as lymph nodes, spleen, liver, epididymis, bone marrow, as well as the placenta, and resides to generate reservoirs. It was documented that the allantoic factors could stimulate the growth of most *Brucella*. These factors include the Erythritol, in addition to possibly steroid hormones and some other substances (4). The response against *Brucella* spp. involves the whole principles of the immune system from innate to adaptive immunity (5).

It was demonstrated that the *Brucella* species has two important heat-stable surface antigens, designated as A and M which are responsible for the action of agglutination. Moreover, it was documented that *B. abortus* has large amounts of A antigen, while the M antigen was in small amounts (5). It was known that there are several serological tests applied for the laboratory testing of the disease. Nevertheless, no single test is convenient in all epidemiological investigations due to some problems which were recorded in sensitivity (*Se*) and/or specificity (*Sp*) (6). Moreover, it was found that the Rose Bengal Plate test (RBPT) was the most sensitive test commonly used; however, it still needs more confirmation with other tests (7).

The course of *Brucella* infection in fact could be affected by both the cell-mediated cytokines and/or antibodies which are involved in the clearance of intracellular bacteria (8). Cytokines play a key role in determining both protective and non-curative immune responses. It has been shown that the *IL-1β* is considered a master regulator of host inflammatory and immune responses to bacterial infection due to its ability to induce the expression of several chemokines and adhesion molecules. Moreover, they are responsible for the stimulation of the phagocytic activities of the white blood cells, especially neutrophils and monocyte, and increase the libration of the reactive oxygen species (9). However, Lacey, Mitchell (10) reported that although both *IL-1* and *IL-18* promoted joint Inflammation, only *IL-18* contributed to protection against *Brucella* infection.

It was also reported that the activation of inflammasome after the infection caused by *B. abortus* in the macrophages will never induce pyroptosis (11). Since the impact of *IL-1β* on *B. abortus* invasion and survival remains elusive, the current study aimed to elucidate the actual roles of these potent cytokines in the modulation of the initial immune response to *Brucella* infection. In light of the aforementioned issues, the present study aimed to detect *Brucella abortus* in the placenta of aborted women and cows and estimate the expression of interleukin 1β (*IL1β*) gene associated with immune response mechanisms to *Brucella abortus* infection.

2. Materials and Methods

2.1. Sampling and Study Population

A total of 100 blood samples were collected from 23 and 30 aborted women and cows, respectively. In addition, 21 and 26 blood samples were obtained from non-aborted women and cows. This study was conducted in different areas of Basrah province, Iraq, from September 2019 to February 2021.

2.2. Sample Processing

The placentas were collected from women and cows immediately after abortion. A part of placental tissues was excised by a sterile scalpel and placed in a sterile tube containing normal saline. These samples were transported to the laboratory under chilled conditions. Each placenta fragment was stored within liquid nitrogen until DNA and RNA extraction. After two weeks of abortion, 5 ml of blood was also re-collected again from aborted and non-aborted women and cows. All samples were directly cooled down using an icebox and left for 20 min to clot. Serum samples were separated by centrifugation at 6000 x g for 5 min and stored at -18°C for further processing in Rose Bengal Test.

2.3. Identification of *Brucella abortus*

2.3.1. Serological Testing by Rose-Bengal Test

The Rose-Bengal test was carried out as described by Padilla Poester, Nielsen (12). Standard *Brucella* antigen was mixed with equal volumes of a serum sample, the result read within 4 min, and the occurrence of agglutination during the first two minutes was considered a positive result. The delayed occurrence for more than 4 min was regarded as a negative result.

2.3.2. Polymerase Chain Reaction Detection of *Brucella abortus*

The genomic DNA was extracted from the placenta by using a DNA extraction kit (Geneaid, Korea). The extracted DNA was quantified using NanoDrop spectrophotometer (Quawell, USA) at a wavelength of 260/280 nm and visualized on 1% agarose gel stained with safety dye (Green-DNA DYE; Biotech, USA) under UV transilluminator (Vilber Lourmal- CE; Taiwan). The ALK-B genes based single polymerase chain reaction (PCR) was performed for amplification of ALK-B gene primers designed by Terzi, Büyüktanir (13)

(F: 5'-GCGGCTTTTCTATCACGGTATTC-3', R: 5'CATGCGCTATGATCTGGTTACG-3'). The PCR mixture consisted of 5.5µL of nuclease-free water, 1µL of each primer, 5µL of DNA template, and 12.5 µL of master mix. The PCR was performed on a thermocycler (Techne; UK) under the conditions: 95°C for 10 min, 94°C for 15 sec, 5 °C for 1 sec, and 72°C for 1 min, as well as 40 sec for elongation and the final extension at 72 °C for 5 min. The cycles were repeated 35×. After gel electrophoresis on 2 % agarose, the expected amplicons with 136 bp for *ALK B* were visualized and photographed under a UV transilluminator.

2.4. RNA Extraction and Reverse Transcription

Total RNA was extracted from the placenta according to the manufacturer's instructions (total RNA isolation kit; Promega/ USA). The extracted RNA was quantified and evaluated spectrophotometrically by using Bio photometer plus (NanoVue, USA). The OD_{260}/OD_{280}. Total-RNA samples were used in complementary DNA (cDNA) synthesis step (RT- step) by Reverse Transcriptase kit provided by Bioneer company/Korea, Briefly: 5 µl of Template RNA, 1µl of Oligo dt 20, and 14 µl of DEPC-D.W. This RT-PreMix was placed in Accu-Power Rocket Script. RT-PreMix tubes contain lyophilized reverse-transcriptase enzyme and are completely dissolved by using a vortex-mixer. The Ribonucleic acid (RNA) was converted into complementary DNA (cDNA) in the thermocycler under the following conditions: Primer annealing (oligo dt 20): 37°C for 10 min, cDNA-synthesis(RT-step): 60°C for 1 h, and Heat-inactivation: 95°C for 5 min. The synthesized cDNA was kept at -20°C.

2.5. Quantitative Polymerase Chain Reaction

Syber green dye-dependent quantitative PCR (qPCR) was carried out according to the manufacturer's instructions (Master Mix kit; Promega GoTaq™ qPCR/ USA). The primers that were used to determine gene expression are displayed in table 1. The *GAPDH* gene was used as an endogenous reference gene. All primers were validated using conventional PCR before being used for quantitative PCR. The PCR reaction mixture (25 µL) was composed of sample cDNA (5µl), each forward and reverse Primers (1µl), PCR master mix(12.5µl), and sterile deionized water(5.5 µl). Amplification was achieved at denaturation at 95°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, and final extension at 72°C for 5 min. After gel electrophoresis on 2 % agarose, only one band for each primer pair with its expected amplicons size was visualized and photographed under a UV transilluminator.

Table 1. Primer pairs used for determining women's cytokines gene expression by the quantitative polymerase chain reaction

Gene (systematic name) primer		Sequence (5'-3')
Human *IL1β*	F	CCACAGACCTTCCAGGAGAATG
	R	GTGCAGTTCAGTGATCGTACAGG
Human *GAPDH*	F	GAGTCAACGGATTTGGTCGT
	R	GACAAGCTTCCCGTTCTCAG
Cow's *IL1β*	F	TCCTCCGACGAGTTTCTGTGTG
	R	GGGATTTTTGCTCTCTGTCCTGG
Cow's *GAPDH*	F	GGCGTGAACCACGAGAAGTATA
	R	CCCTCCACGATGCCAAAGT

The qPCR procedure was performed in triplicates, and the reaction mixture (25 µL) included sample cDNA (5µl), 1µl of each (forward, reverse, and *GAPDH* Primers), qPCR master mix (12.5µl), and sterile deionized water (5.5 µl). The qPCR was achieved at 95ºC for 5 min, followed by 45 cycles of 95°C for 20 sec, 60ºC for 20 sec, and 72ºC for 30 sec. All samples were run in triplicate, and relative quantification was performed by using the $2^{-\Delta\Delta Ct}$ method. The amplification efficiencies for each pair of PCR primers were determined by producing standard curves using a series of tenfold dilutions of the conventional PCR products for each gene. The conventional PCR products were purified using the (Promega Wizard ® SV Gel) and (PCR Clean-Up System) before they were used to generate the standard curves. The amplification efficiencies for all primer sets were ≥ 0.90, indicating that the amplifications were highly efficient. To confirm that, only one PCR product was obtained for each set of the primers, and melt-curve analysis was performed at the end of each Real-time PCR. This involved heating the reaction mixture to either 65°C or 72°C for 45 sec and then raising the temperature by 1°C every 5 sec until a maximum temperature of 95°C was reached. Moreover, the change in the fluorescence was measured at the same time. In all cases, there was only one peak in the fluorescence curve. This confirmed the agarose-gel results which had shown that only one product was produced for each PCR primer pair.

2.6. Statistical Analysis

Statistical analysis was performed in SPSS software (version14). To demonstrate any association, the exact Fisher test and *t*-test were used with the limit of significance being set at 5%. The values of OR (odds ratio) and 95% CI (confidence interval) were calculated. Serological and molecular tests were compared with each other. Sensitivity, specificity, as well as positive and negative predictive values, were used to assess the diagnostic efficacy of RBT and *AlkB*- based PCR.

3. Results

3.1. Distribution of *Brucella abortus* in Aborted Women and Cows

The detection of *Brucella abortus* was performed by RBT and PCR-based *AlkB gene (AlkB*-PCR) in the sera and placentas samples of aborted women and cows, respectively. The RBT results revealed that out of 23 tested aborted women, 3 (13.1%) serum samples were positive. In the case of sera from aborted cows, 9 (30%) out of 30 samples were RBT positive. Negative RBT results were observed in all non-aborted women (n=21) and cows (n=26). The results of *AlkB*-PCR showed a successful binding between extracted DNA and specific primers targeting *AlkB genes*. The placentas of all non-aborted women (n=50) and cows (n=50) appeared with negative *AlkB*-PCR results. The overall prevalence rates of *Brucella abortus* infection were 13.1% and 5% as determined by RBT and *AlkB*-PCR in aborted women's sera and placentas samples, respectively. The overall prevalence rates of *Brucella abortus* infection in the sera and placentas from aborted cows were 30% and 11% as estimated by RBT and *AlkB*-PCR, respectively. The statistical comparison of individual diagnostic test outcome proportions revealed that there was no significant difference between the outcome proportion of RBT and *AlkB*-PCR in aborted women's sera and placentas (($\gamma^2 = 1.998$: 1df: *P=0.158*; 95% CI=-2.433-27.405). In the case of sera and placentas from aborted cows, the outcome proportions of the two diagnostic tests were significantly different ($\gamma^2 = 6.350$: 1df: *P=0.0117*; 95% CI=3.636-37.495) (Table 2).

3.2. Association between *Brucella Abortus* and Abortion According to Rose Bengal Test

The association between *Brucella abortus* and abortion was tested in aborted women and cows using chi-square odd ratio statistics tests. The results of RBT illustrated that *Brucella abortus* infection was associated with abortion (γ^2 = 5.543; 1df; *P=0.02*) (Tables 3). According to the odd ratio calculation, this association was statistically significant; therefore, an abortion caused by *Brucella abortus* infection occurred

6.2 times higher in seropositive (OR=6.2; CI= (1.19 to 31.82); *P=0.03*), compared to seronegative cows. In a similar vein, aborted women were investigated by Chi-squared test, and the association between *Brucella abortus* infection and abortion was not statistically significant (γ^2=0.341; 1df; *P=0.56*). Nevertheless, the abortion caused by *Brucella abortus* was 1.8 times higher in seropositive, compared to seronegative aborted women (OR=1.8; CI= 0.26-11.66; *P=0.56*). Moreover, 2 (8.7%) aborted women **a**nd 2 (7.1%) aborted cows showed false-negative RBT results (Table 3).

Table 2. Outcomes of the individual diagnostic test of *Brucella abortus* in aborted women and cows

Source of Samples	Sample/test	Tested n.	Test results		Chi-squared test		
			Positive n (%)	Negative n (%)	Chi-squared value	*P- value*	95% CI*
Aborted women	Serum/RBT Placenta/PCR	23 100	3 (13.1) 5 (5)	20 (86.9) 95 (95)	1.998	*0.158*	-2.433 to 27.405
Aborted cows	Serum/RBT Placenta/PCR	30 100	9 (30) 11(11)	21(70) 89 (89)	6.350	*0.0117*	3.636 to 37.495

CI *: Confidence interval

Table 3. Association between *Brucella abortus* and abortion in women and cows according to Rose Bengal Test

RBT	Aborted women n (%)	Non-aborted women n (%)	Total n=44	Chi-square statistic *P-value*	OR	95 % CI
Positive	3 (14.3)	18 (85.7)	21	0.56	1.8	0.26-11.66
Negative	2 (8.7)	21 (91.3)	23			
RBT	**Aborted cows n (%)**	**Non-aborted cows n (%)**	**Total n=56**	**Chi-square statistic *P-value***	**OR**	**95 % CI**
Positive	9 (32.1)	19 (67.9)	28	0.02	6.2	1.19-31.82
Negative	2 (7.1)	26 (92.9)	28			

OR= Odds ratio, CI= Confidence interval, RBT: Rose Bengal Test

3.3. Association Between *Brucella abortus* and Abortion according to *Alkb*- Based Polymerase Chain Reaction

Figure 1 demonstrates a successful binding between placental DNA extraction and specific primers targeting *AlkB genes* in *Brucella abortus*. Under the U.V light, PCR products corresponding to *AlkB* (136bp*)* were observed in 5 (5%) aborted women and 11 (11%) cows. All non-aborted women showed negative results for *AlkB genes*. The association between *Brucella abortus* and abortion was investigated using a Chi-squared test. It was found that *Brucella abortus* was associated with abortion in the case of aborted cows (γ^2 = 5.94; 1df; *P= 0.015*). The risk of abortion was 12.98 times higher in PCR-positive compared to non-aborted cows (OR=12.98; CI= (0.749-224.896); *P=0.078*). For aborted women, *Brucella abortus* was considered to be not statistically associated with abortion (γ^2 =2.59; 1df; *P=0.108*) (OR=5.82, CI= 0.315 to 107.33, *P=0.24*) (Table 4).

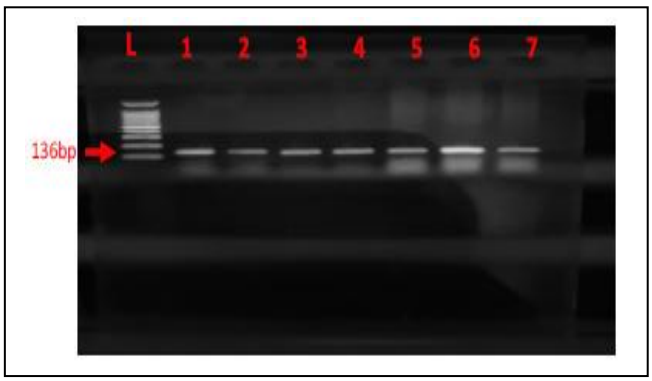

Figure 1. Polymerase chain reaction amplification of *Brucella AlkB* gene (136bp)
The PCR amplification was run on 1.5% agarose gel stained with Safety day. Lane: L, 100 bp DNA ladder marker; Lane (1- 7) *Brucella* abortus *AlkB* gene-positive results

Table 4. Association between *Brucella* abortus and abortion in women and cows according to AlkB based Polymerase Chain Reaction

AlkB-PCR	Aborted women n (%)	Non-aborted women n (%)	Total	Chi-square statistic *P-value*	OR	95 % CI
PCR+	5(5)	95(95)	100	0.108	5.82	0.32-107.33
PCR-	0	50(100)	50			
Test	**Aborted cows n (%)**	**Non-aborted cows n (%)**	**Total**	**Chi-square statistic *P-value***	**OR**	**95 % CI**
PCR+	11(11)	89 (89)	100	0.015	12.98	0.75-224.896
PCR-	0	50(100)	50			

OR: Odds ratio, CI: Confidence interval

3.4. Comparison between Rose Bengal Test and *Alkb*-Based Polymerase Chain Reaction

Out of 5 positive *AlkB*-based PCR aborted women's sera, only 3 were positive and 39 samples were negative to RBT. Moreover, two false-negative results were observed in each aborted woman and cow. All non-aborted women and cows (21 and 26, respectively) were correctly identified by the RBT as true negative (Table 5). Using Med-Calc statistical software and considering *AlkB* based PCR as the gold standard, the sensitivity, specificity, and accuracy of RBT were calculated as 60.00%, 53.85%, and 54.55% respectively, with a 95% confidence interval (CI) of 14.66%-94.73%, 37.18%-69.91%, and 38.85%-69.61%. Furthermore, the positive likelihood ratio (PLR) for the RBT test was 1.30, while the negative likelihood ratio (NLR) was determined as 0.74. For RBT, a positive predictive value of 14.33% was reported, while the negative predictive value was reported as 91.28% (Table 6).

Regarding RBT for sera samples of aborted cows, only 9 samples from 11 positive PCR were positive, while 45 samples were negative. The test sensitivity was 81.82% with a 95% CI of 48.22%-97.72%, and the specificity was 57.78% with a 95% CI of 42.15%-72.34%, in addition to an accuracy of 62.49% with a 95% CI of 48.54%-75.07%. The positive likelihood ratio for the RBT test was evaluated at 1.94, while the negative likelihood ratio was determined at 0.31. The positive predictive value was reported as 32.08%, while the negative predictive value was obtained at 92.88%. The results of the current study indicated that RBT is superior in sensitivity and specificity in the case of cows, followed by women (Table 6).

3.5. *IL1β* Gene Expression according to the Quantitative Polymerase Chain Reaction

According to $2^{-\Delta\Delta}$Ct method, the data were presented as a fold change in gene expression. The mean of *IL1β* gene expression was higher in aborted women and cows (114.905±99.661 and 22.454±18.528, respectively), compared to non-aborted women and cows (4.953±5.564 and 2.033±1.845, respectively) (Table 7; Figures 2 and 3). Table 8 displayed statistical analysis of a fold change in *IL1β* gene expression of aborted women and cows. The fold change in *IL1β* gene expression was higher in aborted women (114.905±99.661), in comparison with that in aborted cows (22.454±18.528); nonetheless, this difference was not considered to be statistically significant (*P>0.05*).

Table 5. Raw data obtained from Rose Bengal Test (RBT) where true positives= (a), true negatives= (d), false positives= (b) and false negatives= (c)

Women		*Brucella* (Rose Bengal Test)		total
		Positive	**Negative**	
AlkB-PCR	Positive	3^a	2^b	5 (a+b)
	Negative	18^c	21^d	39 (c+d)
Cows				
AlkB-PCR	Positive	9^a	2^b	11 (a+b)
	Negative	19^c	26^d	45 (c+d)

Table 6. Statistical evaluation of *Brucella abortus* diagnostics test in aborted women and cows

Statistic	Women		Cows	
	Value	95% CI	Value	95% CI
Sensitivity	60.00%	14.66%-94.73%	81.82%	48.22%-97.72%
Specificity	53.85%	37.18%-69.91%	57.78%	42.15%-72.34%
Positive likelihood ratio	1.30	0.59-2.87	1.94	1.25-3.01
Negative likelihood ratio	0.74	0.24-2.26	0.31	0.09-1.13
Disease prevalence	11.40%		19.60%	
positive predictive value (*)	14.33%	7.04%-26.97%	32.08%	23.31%-42.34%
Negative predictive value (*)	91.28%	77.48%-96.95%	92.88%	78.40%-97.91%
Accuracy (*)	54.55%	38.85%- 69.61%	62.49%	48.54%-75.07%

(*) These values are dependent on disease prevalence

Table 7. Relative gene expression of *IL1β* gene in the placentas from women and cows

Clinical status	Mean±SD	95% CI	SE	*P*-value
Aborted women n=5 Non-aborted women n=5	114.905±99.661 4.953±5.564	-212.8901 to -7.0139	44.639	0.0391
Aborted Caws n=5 Non-aborted Cows n=5	22.454 ±18.528 2.033±1.845	-39.62 to -1.22	8.327	0.0398

SD: Standard Deviation, CI: Confidence interval, SE: Standard Error

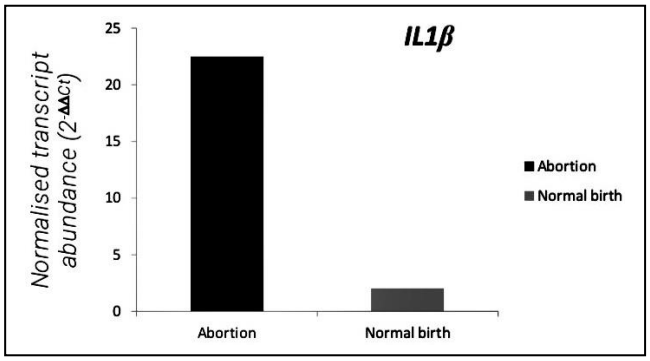

Figure 2. Relative expression of *IL1β* genes in the placenta of Cows
Using the 2 $^{-\Delta\Delta}$Ct method, the data are present as a fold change in gene expression normalized to an endogenous reference gene (*GAPDH*) and relative to the samples from naturally parturated cows

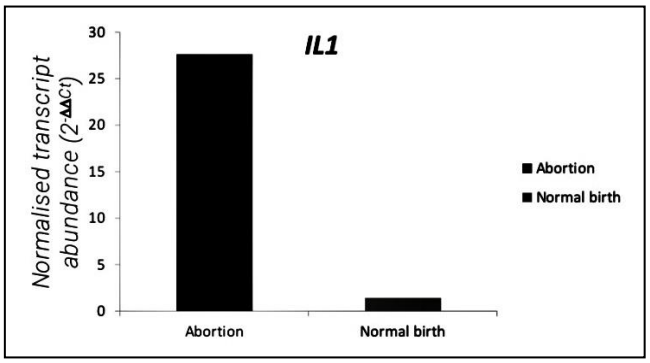

Figure 3. Relative Expression of *IL1β* Genes in the Placenta from Women
Using the 2 $^{-\Delta\Delta}$Ct method, the data are presented as a fold change in gene expression normalized to an endogenous reference gene (*GAPDH*)

Table 8. Comparison of relative gene expression of *IL1β* gene between the placentas from cows and women

Clinical status	Mean±SD	95% CI	SE	*P*-value
Aborted women n.=5	114.905±99.661	-12.0881 to 196.9901	45.333	0.0757
Aborted Cows n.=5	22.454 ±18.528			

SD: Standard Deviation, CI: Confidence interval, SE: Standard Error

4. Discussion

In developing countries, such as Iraq, several conventional tests, which mostly use milk or serum, are applied for the diagnosis and screening of *brucellosis* (14-18). To the best of our knowledge, this is the first study in Iraq to detect *B. abortus* DNA in the placentas of women and cows. The *Brucella abortus* was not isolated in this study, and since *Brucella* culturing is hazardous, the isolation rate is very low even in experienced laboratories (19). When *B. abortus* presents as a few organisms in the samples or when the samples were heavily contaminated, the probability of successful isolation of the bacterium will be reduced. However, strong seropositive results with clinical presentation confirmed the final diagnosis of the disease.

Moreover, the serological confirmative diagnosis could fail since the antibody titers against the bacterium will rise defiantly after one to two weeks after the infection (12). Nevertheless, the molecular technique will be the fit diagnostic procedure to detect the circulating DND of *Brucella.* Those facts could clarify the absence of antibodies in 20 and 21 serum samples of women and cows, respectively. The serological diagnosis of *brucellosis* is a hypothetical guide to the infection; however, the exact laboratory verification of the microorganism needed the isolation of causative bacteria or detection of its DNA using PCR. Therefore, the diagnostic approaches to the disease via serological detection must be completed by bacteriological and/ or molecular techniques (20). The PCR analysis was able to identify *Brucella* DNA in negative serum samples of animals, and it was suggested to apply the PCR even as a tool for the routine diagnosis of the disease. The current results of *AlkB*-based PCR corroborate this proposal since 2 (8.7 %) women **a**nd 2 (7.1%) cows of RBT seronegative showed positive PCR results.

Based on RBT, the current total prevalence rates of the disease in aborted women and cows were 14.3 % and 32.1% respectively. It should be clarified that preventive vaccinations against brucellosis in Iraq have not been precisely found in cows for the last two years; therefore, the current result might reflect natural infection. Several studies have been carried out on brucellosis seroprevalence in Iraq (21). In Mosul, Terzi, Büyüktanir (13) reported a seroprevalence rate of 5.8% in cattle. Al-Alo and Mohammed (14) found that 5.81% of cows were seropositive in Al-Najaf province. In addition, in Basrah province, Hasoon and Al-Amery (17) reported that 27 (10.8%) buffaloes were seropositive in RBT. Probert, Schrader (22) documented that serological tests were different in their diagnostic ability to identify the specific antibodies in a particular immunoglobulin class. Therefore, diseased animals could or could not produce all detectable quantities of antibody isotypes.

This disease in humans is generally caused by *Brucella melitensis* and/or *Brucella abortus*, however, the main pathognomonic characterization is the inflammation of the genitals and fetal membranes, sterility, abortions, and lesions in the joints, as well as the lymphatic system (23). Moreover, apart from spontaneous miscarriage, intrauterine fetal death, especially during the first trimester, was also reported among pregnant women (23, 24). The results indicated that the percent seroprevalence of the disease in a diseased aborted woman was 14.3%, referring to an old and new exposure to the infection with *Brucella* since vaccination trials and programs against the disease for human beings have not been carried out in all parts of Iraq.

The incidence of *Brucella*-related abortion in women is not documented in Iraq despite several reports on the disease in cattle. Since brucellosis has been associated with abortion, complications during pregnancy related to the disease in this country need further consideration. In one study, it was pointed out that in Basrah, Iraq, a potent relationship was reported between the complication of genital tracts of humans and diseases in animals. It was also indicated that 77/92 (83.7%) sterility patients were seroreactive for *Brucella* spp of both *B. melitensis* and *B.abortus*, and DNA has been indicated by PCR in 59/73 (80.82%) and 2/73 (2.74%) of these patients (16).

In earlier studies in Iraq, RBT was positive in 71.3% of cases (25). In the city of Mosul, 115 (29.9%) cases showed a positive reaction in RBT (26). In a similar vein, the seroprevalence of 8.6% by RBT was recorded in the Erbil governorate (27). In Jordan, seroprevalence among women with miscarriage was 1.8% (28). The PCR amplification targeting species-specific gene *alkB* has been applied to assure the involvement of the DNA of the organism in placentas samples. The detection of an amplicon of 136 bp indicated the existence of the DNA of *Brucella abortus*. In the same way, *AlkB*-based PCR was used by Terzi, Büyüktanir (13) and Probert, Schrader (22).

In the current study, 5 (5%) aborted women's placenta samples were PCR positive, and 11(11%) samples originating from aborted cows were positive in PCR. Even being aborted, the samples originating from 95/100 (95%) aborted women's placentas and 89/100 (89%) placentas from aborted cows were negative in *AlkB*- PCR. These results might be explained by the study by Terzi, Büyüktanir (13) who indicated that the detection of the DNA of the *Brucella* by PCR technique is impossible in placenta samples obtained from aborted women and cows when the disease is present in a chronic form.

It has been found that some samples of placentomes might have an organism below the detection limit and fail to be detected as positive. Furthermore, 2 (8.7%) aborted women **a**nd 2 (7.1%) aborted cows of RBT seronegative showed positive results by *Alkb*- PCR. This result might be attributed to an explanation provided by Gwida, El-Ashker (29) who reported that positive cattle with positive *Alkb*-PCR could reflect an infection by Brucella field strain type, whereas, the positive *Alkb*-PCR results from negative animals due to the circulation of RB51 vaccine DNA in vaccinated animals or the circulation of field strain in diseased animals before seroconversion. On the contrary, Sarker, Begum (30) found that out of 14 RBT milk samples from positive cows, 3/14 (21.43%) cases were *Alkb*-PCR positive. The confirmatory diagnostic tests and screening methods are considered the perfect tools for active epidemiological analysis (31).

In Iraq, particularly in Basrah province, numerous studies have been conducted to estimate the prevalence and incidence of bovine brucellosis in different animal species. Nonetheless, the sensitivity and specificity of the serological test data are not available. Understanding the specificity and sensitivity of any test analysis could decrease the problems of diagnosis in both infected and non-infected animals to avoid unnecessary economic problems when the diseased animals are faultily recognized by the tests (32). According to the current results, it was indicated that the RBT had a remarkable achievement in aborted cows, as well as a descending order of sensitivity, specificity, and finally, accuracy in aborted women. The RBT may represent the most popular and ideal examination as a screening tool due to its fast result and low-cost availability (33).

The use of a proper and suitable test analysis with a positive predictivity is too important to guarantee the presence of disease, while a specific test with good negative productivity excludes the infection (34). The purpose of the diagnosis is to find cases while preserving false-positive results to a minimal level. One of the most important impediments of the RBT is a major number of false-positive results which could be described as a result of the cross different reactions of the test to many types of bacteria. In contrast with this finding, the present results revealed that all seropositive aborted women and cows illustrated positive results when they were tested with *Alkb*-PCR positive.

Regarding PCR as the gold standard, the present study indicated that the sensitivity values of RBT were 60.00% and 81.82% in aborted women and cows, respectively. In this respect, Zakaria (35), Černyševa, Knjazeva (36), and Ruiz-Mesa, Sanchez-Gonzalez (37) reported the sensitivity of the RBT as 79 %, 68.6%, and 93.8, respectively. It was documented that the sensitivity and specificity of any serological tests could be affected by some external environmental effects,

such as the temperature conditions when the test is performed, the epidemiological state of the disease, the level of vaccination in animals, and the presence of cross-reactive antibodies from other Gram-negative bacteria which share similar epitopes with *Brucella* spp. (6).

There is evidence that the intracellular multiply of the causative microorganism in the trophoblastic cells is more important in the pathogenic effect of *B. abortus* to stimulate the abortion and placental inflammation in animals (38). The capability of the causative organism to the residence in the body tissues and cause a chronic infection will be related to its strength to survive in the macrophages by interfering with phagolysosomal fusions. Macrophages will be inducted by interleukin-1L (IL-1β) and granulocyte-macrophage-colony-stimulating factor (GM-CSF).

Fernández, Ferrero (39) proposed that the trophoblasts of humans might supply the environment of any local inflammation during *B. abortus* infections either via a direct reaction to the causative pathogen or even interactions with monocytes/macrophages or neutrophils, potentially contributing to the pregnancy complications of brucellosis. The *IL1 β* is a proinflammatory cytokine which plays an important role in inflammatory response stimulation and induces the expression of many cytokines and chemokines, such as interleukins (*IL2*, *IL8*, and *IL12*), production of tumor necrosis factor (TNF-alpha), interferon (IFN-γ). Moreover, it contributes to the promotion of apoptotic cell death in fetal membrane tissues, and all the aforementioned effects are considered harmful to fetal development (40).

According to qPCR results, the mean of *IL1β* gene expression was higher in aborted women and cows, compared to that in non-aborted women and cows. This result is in agreement with those obtained by Ali and Abdelwahab (41) who found significant increases in IL-1β levels in *B. abortus*-infected cows, in comparison with the control group. In addition, the experiments *in vitro* proved that the causative organism induces human and murine monocytes to liberate proinflammatory cytokines, such as TNF-α and IL-1β, where TNF-α is a co-stimulator in the production of IFN-γ. The results of the current study are also supported by Priyanka, Shringi (42) who reported that peripheral blood mononuclear cells (PBMCs) from serologically *Brucella*-positive diseased cows inherently exhibited a different cytokine gene expression profile, compared to the uPBMCs from healthy serologically negative control cows.

As per the findings of the present study, it can be concluded that RBT can be routinely used for the diagnosis of *Brucella* as a cost-effective, sensitive, and accurate test. Nevertheless, PCR is recommended as a gold test for the identification and differentiation of *Brucella* infection detection in aborted women and cows. It was found that the association between *Brucella abortus* and abortion in cows was statistically significant. *Brucella* infection of the placentas led to marked changes in immune responses through the increment of *IL1 β* expression which induces the expression of many cytokines and chemokines that are considered harmful to fetal development and lead to consequent abortion.

Authors' Contribution

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

Ethics

Approval for the research study was obtained from the University of Basrah, Basrah, Iraq ethics board (project approval number 15784000).

Conflict of Interest

The authors declare that they have no conflict of interest.

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