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Detection of *Brucella abortus* in Cows Suffering From Some Reproductive Disorders

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THIS investigation was undertaken to detect the *Brucella abortus* in cows with some reproductive disorders. Antibody and DNA for *Brucella* spp. were tested in serum and placentas of cow using Rose Bengal Test (RBT) and polymerase chain reaction (PCR) respectively. Odds ratio(OR) and chi square (χ^2) tests were done to determine the statistical association between the results. Depending on clinical samples , RBT and *16s rDNA*-based PCR data showed an overall percentage 30% (95% CI= 0.147-0.494) and 11% (95% CI= 0.056-0.288), respectively, Placenta retention was not significantly associated with RBT and PCR positivity (OR=1.40, 95% CI= 0.224- 8.768, χ^2=0.130 , df =1, p =0.7) and (OR=0.33, 95%CI =0.057-1.857, χ^2=1.741, df=1,p =0.2) respectively. RBT seropositivity in cows with retained placentas was 25% (95 % CI= 0.032 - 0.651), while the percentage of those who did not was 31.8 % (95 % CI= 0.139 - 0.549).*16s rDNA* and *AlkB* based -PCR positivity in placenta retention was 25% (95 % CI = 0.032–0.651), compared to the cows without Placenta retention , 9.8 % (95 % CI = 0.046-0.178). PCR based *16s rDNA* and *AlkB* positivity was 9 % with 95%CI (0.042 - 0.164)in RBT positive aborted cows. while 2% (95%CI: 0.002 to 0.070) of PCR positivity was detected in RBTnegativeaborted cows.

Keywords: *Brucella abortus*, Abortion, Retention of the placenta,*16s rDNA*.

Introduction

Brucellosis is a highly contagious disease neglected sometimes in a few areas, however, its recent epidemiological manifestations including its occurrence in new regions besides its fast transmission from animals to humans, are of great value [1-4].

In animals, especially sexually mature females, Brucellosis induces and causes abortion with stillbirth, retention of the placental membranes, metritis, as well as drop-in milk production, Whereas, in males, brucellosis plays a major role in causing orchitis and epididymitis. However, Infertility could be seen in both sexes [5]. Abortion is an early and premature ejection of the dead embryo that mostly occurs because of some abnormalities of the reproductive system [6].As this un abnormal pathological reflex (the abortions) could lead to massive economic losses in diseased animals and could be terminated with food insecurity [7]. Abortion is an obvious sign induced bymulti-factorial which might include infectious causes and nutritional deficiencies beside other extra factors, As, It has been documented that the Infectious causes of abortion could be responsible for more than 90.0% of ruminants abortions, including, the viruses, bacterial agents, protozoans as well as the fungi, Further, It was known that among all

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the major pathogens, Brucella was the main cause of abortion in farm animals[8]. *Brucella abortus*. Is the important infectious microorganism responsible for causing abortions mainly at the end period of pregnancies in animals[9,10].

It has been mentioned that, the isolation of the causative Brucella spp. using its specific media is still the gold standard technique for an accurate diagnosis of this bacteria,despite it could not be succeeded always and reflected major hazard of infection for the laboratory works and isolation of *Brucella* spp. was tedious, time-consuming, and difficult due to the intra-cellular and fastidious nature of the bacteria [11]. Rose Bengal is considered as the common serological test used for brucellosis fast diagnosis, However, the Serum tube agglutination test was also another accurate test used, On the other hand, the development of molecular biological techniques (PCR) was used successfully detection of *Brucella* DNA[12, 13]. Because of the importance of brucellosis and its highly spread, this study was conducted on cows to detect the *Brucella abortus* with a history ofreproductive disorders including abortion and retention of placenta.

Material and Methods

Sampling

Thirty aborted cows' blood samples and 26 blood samples from cows that weren't aborted were collected. A total of 100 placenta samples were taken from an aborted cow, including 8 samples from cows who had retained placentas. In addition, 50 placentas were collected from non-aborted cows. This study was carried out in various areas of Basrah province between September 2019 and February 2021. Approximately 5ml of blood was collected from each individual cow. Blood samples were immediately chilled with an ice box and allowed to coagulate for 20 minutes. After that, the serum from blood samples were extracted and centrifuged for 5 minutes at 6000 rpm to utilize for the Rose Bengal test.

The placentas were taken from cows immediately after an abortion, and a part of the placental tissues was excised with a sterile scalpel and placed in a sterile tube,then fragments of the placenta were straightaway put into liquid nitrogen, until DNA extraction.

Rose Bengal Test (RBT)

The kit of Rose Bengal test (Lorne, UK) was used as followed by the Office International des Epizooties (OIE) standard procedure[14]. As, antigen and serum samples were brought to room temperature and 25 µl of each serum sample was mixed with an equal volume of antigen using a clean splint of wood,then shaken nicely for min(at room temperature) before being examined for agglutination. After min, RBT-positive samples showed evidence of agglutination.

Extraction and purification of DNA.

Genomic DNA wasextracted from all samples of the placenta using a DNA extraction kit (Geneaid, Korea) according tothe manufacturer's instructions. The extracted DNA was quantified using NanoDrop spectrophotometer (Quawell, USA) at the wavelength of 260/280 nm and visualized on 1% agarose gel stained with safety dye (Green-DNA DYE, Biotech, USA) under UV transilluminator (BioRad, USA).

PCR detection of Brucella abortus

Using a thermocycler (Bio-Rad, USA), PCR amplification of the *16S rDNA* and *Alk-B* genes was performed on genomic DNA from placenta samples (Techne, UK). Under the UV transilluminator and gel documentation system, the amplicons of 800 and 136 bp for *16s rDNA* and *Alk-B* were visualized and photographed. Primers used for amplification were the universal *16s rDNA* forward (5-GTGCCAGCAGCCGCCGTAATAC-3) and reverse (5-TGGTGTGACGGGCGGT GTGTACAAG-3) primers according to [15]. The expected PCR product was 800 base pairs. DNA template (5 µl) was added to a final reaction volume of 25 µl consisting of 12.5 µl of one Taq2x master mix, 1 µl (10pmol/µl) of each primer, and 5.5 µl of nuclease-free water. PCR reaction was run at an initial denaturation of 95 ^{O}C for 5 min, followed by 40 cycles of denaturation at 95 ^{O}C for 30 s, annealing at 52 ^{O}C for 30 s, extension at 75 ^{O}C for 90 s, and a final extension at 75 ^{O}C for 5–60 min. The PCR products were visualized in a 2% agarose gel after electrophoresis at 110 volts for 60 min.

The *Alk-B* genes based (PCR) was performed for amplification of *Alk-B* forward (5'-GCGGCTTTTCTATCACGGTATTC-3)' and reverse (5'CATGCGCTATGATCTGGTTA CG-3') primers designed byTerzi et al.[16]. PCR mixture consisted of 5. 5µl of nuclease-free water, 1µl(10pmol/µl) of each primer, 5µl of DNA template, and 12.5 µl of master mix. PCR was performed on a thermocycler (Techne, UK) under the conditions: 95$^{°C}$ for (10 min), 94$^{°C}$ for (15 sec), 57$^{°C}$ for (1 sec), and 72$^{°C}$for (1 min and 40 sec) for elongation. Cycles were repeated 35×. And the final extension was 72 °C for 5 min. After gel electrophoresis on 1.5 % agarose the expected amplicons with 136 bp for *Alk-B* was examined.

Statistical analysis

MS Excel version 2016 was used to collect and clear up all the data. The Chi-square ($\chi 2$) and odds ratio tests were employed to see if there was any correlation between the results, at a 5% significance level.

Results

RBT and PCR for the detection of Brucella species

RBT and *16s rDNA*-based PCR data showed that the overall RBT and PCR positivity of *Brucella*spp. in aborted cows was 30% (0.147-0.494) and 11% (0.056-0.288), respectively, depending on the type of clinical samples (Table 1).

Table (2) displays the results of the Rose Bengal Test for Reproductive Disorder in cows. The percentage of seropositive cows who had abortions and retained their placentas was 25% (95 % CI= 0.032 - 0.651), while the percentage of those who did not was 31.8 % (95 % CI= 0.139 - 0.549).

To determine whether the reproductive problem was connected to RBT and PCR positive results, the odds ratio and chi-square calculation was utilized. RBT. Table (2), showed that RBT seropositive results were shown to be 1.40 times more common in aborted cows without placenta retention, with no statistically significant correlation. (OR=1.40, 95% CI= 0.224- 8.768, χ^2=0.130 ,df =1, p =0.7).

Table (3). listed *16s rDNA* and *Alk-B* based PCR results for reproductive disorders in cows, in which the percentage of PCR positive results in cows who had abortions and retained their placentas was 25% (95 % confidence interval CI = 0.032–0.651), compared to the percentage of those who did not, was 9.8 % (95 % CI = 0.046-0.178).

The *16s rDNA*-based PCR positivity was 9 % with 95% CI (0.042 - 0.164)in RBT-positive aborted cows. While **2**%(**95%**CI:0.002 to 0.070) of PCR positivity was detected in RBT-negativeaborted cows without placenta retention. *Alk-B* -PCR was used as a confirmatory diagnosisfor *Brucella abortus* in aborted cows' placentas. All *16s rDNA* (800bp) based PCR positive samples showed positive *Alk-B* (136bp) based PCR results (Table 4, Figures1, 2). Odds ratio and chi-square calculation were performed to determine whether PCR positivity was related to RBT results (Table 4). The odds of *Brucella* PCR positive results were 4.85 times higher in animals with RBT positive results compared with those with negative results and this association was considered to be statistically significant (OR= 4.85, 95% CI =1.020 -23.028,χ^2=4.714,df=1,p =0.03).

TABLE 1. The overall RBT and PCR positivity of *Brucellaspp* depending on tested samples.

Tested samples	n/N	%	95% Confidence intervals
Serum	9/30	30	0.147 - 0.494
Placenta	11/100	11	0.056 -0.188

TABLE 2. Rose Bengal Test results in cows according to Reproductive disorder.

Reproductive disorder	Rose Bengal Test positivity		Statistical analysis				
	n/N	% (95% CI)	Odds Ratio (95% CI)	Odds Ratio p-value	χ^2	χ^2p-value	df
Abortion without Retention of placenta	7/22	31.8(0.139- 0.549)	1.40(0.224- 8.768)	0.4	0.130	0.7	1
Abortion with Retention of placenta	2/8	25 (0.032 - 0.651)					

χ^2 =Chi-square, χ^2**p=** Chi-square proportion, **df=** degrees of freedom

TABLE 3. *16s rDNA* and *Alk-B* based PCR results in aborted cows according toreproductive disorder:

Reproductive disorder	PCR +ve		Statistical analysis				
	n/N	% (95% CI)	Odds Ratio (95% CI)	Odds Ratio p-value	χ^2	χ^2p-value	df
Abortion without retention of placenta	9/92	9.8 (0.046-0.178)	0.33 (0.057-1.857)	0.1	1.741	0.2	1
Abortion with retention of placenta	2/8	25 (0.032-.651)					

χ^2 =Chi-square, χ^2p= Chi-square proportion, df= degrees of freedom.

TABLE 4. PCR based *16s rDNA* and *Alk-B* results related to RBT in aborted cows.

PCR results	PCR positivity		Statistical analysis				
	n /N	%(95%CI)	Odds Ratio (95% CI)	Odds Ratio p-value	χ^2	χ^2p-value	df
RBT-positive abortedcows	9/100	9 (0.042-0.164)	4.85 (1.020-23.028)	0.03	4.714	0.03	**1**
RBT negative abortedcows	2/100	2 (0.002-0.070)					

χ^2 =Chi-square, χ^2p= Chi-square proportion, df= degrees of freedom

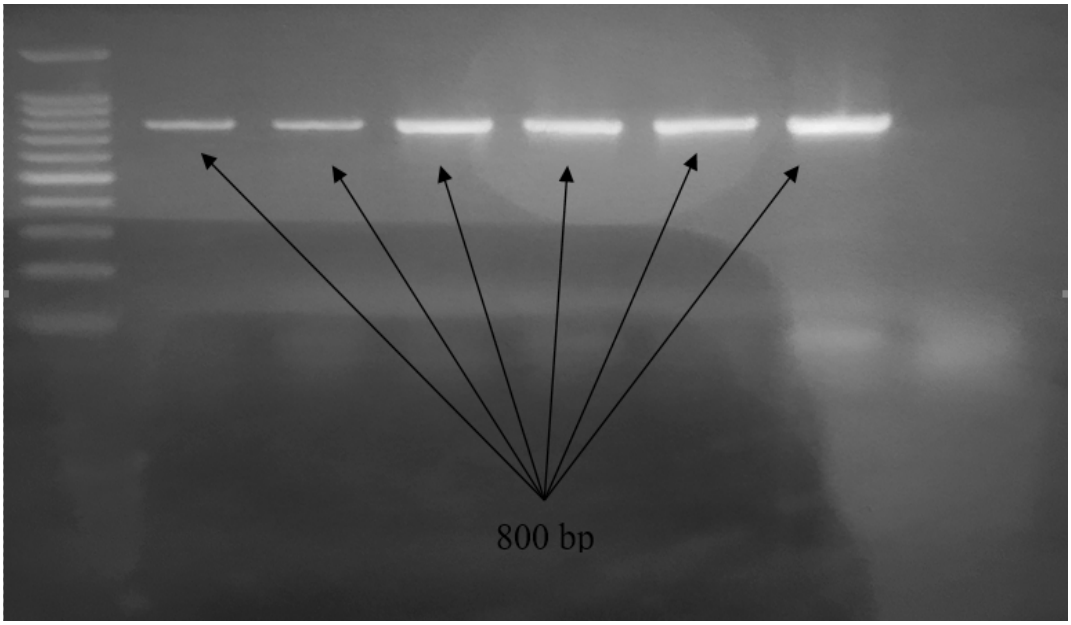

Fig. 1. PCR amplification of *Brucella*sp *16s rDNA* gene (800 bp). PCR amplification was run on 2% agarose gel stained with Safety day. Lane: L, 100 bp DNA ladder marker, Lanes 1-6*Brucella* sp *16s rRNA* gene-positive, Lane 7 negative result.

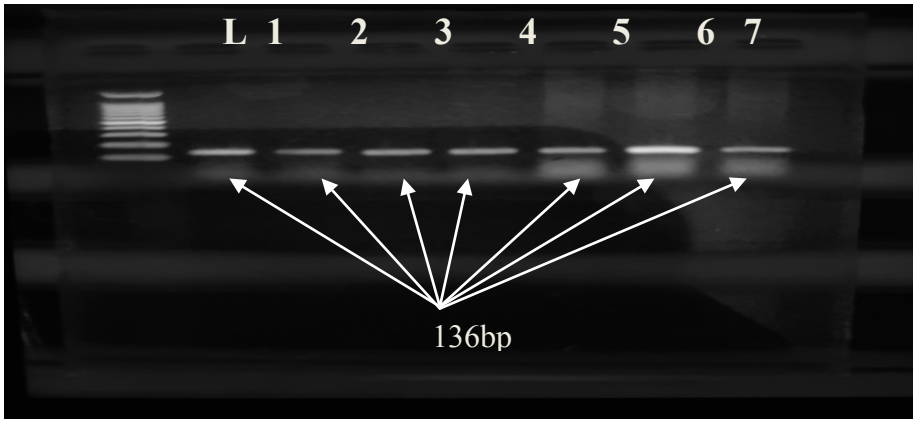

Fig. 2. PCR amplification of *Brucella abortus Alk-B* gene (136bp). PCR amplification was run on 1.5% agarose gel stained with Safety day. Lane: L, 100 bp DNA ladder marker, Lanes1- 7*Brucella* abortus *Alk-B* gene-positive results.

Discussion

Iraq has a high prevalence of both human and animal brucellosishas been indicated [17-20]. Bovine brucellosis can be diagnosed using a variety of serological tests, which complicates the diagnosis for several reasons. However, there is a restriction to the tests, when the organism is intracellular, the disease enters a chronic stage, and the antibody titers may decrease [21,22]. The goal of the current investigation was to use PCR to identify *Brucella* spp. in aborted cow placentas. According to this study, there were 4.85 times greater odds of *Brucella* PCR-positive results in cows with positive RBT results than in cows with negative results. This result supports the findings of Akoko et al.[22] who found that RBT positivity was a great indicator of *Brucella* PCR positivity in livestock. RBT is a suitable test to use for brucellosis diagnosis and may be a helpful screening test, according to previous studies[23]. However, despite being complementary, these two tests identify various infection signs and provide information that overlaps but is distinct [24]. Due to the lower sensitivity of the Rose Bengal Test as seen in the current work and prior investigations [25,21].It was shown that ELISA as well as Fluorescence Polarization Assay, Slow Agglutination Tests, and the Complement Fixation Tests must be taken into consideration in addition to RBT [26]. But in this investigation, *Brucella* DNA was isolated from placenta samples of aborted cows and used in PCR analysis to identify the pathogen. For the quick and accurate detection of *Brucella* spp., PCR methods have been suggested [18,27].

Placentitis and abortion occur in pregnant cows infected with *B. abortus*[28]. Even though there is proof to support the idea that *B. abortus* intracellular proliferation within trophoblastic cells is significant in the pathophysiology of *B. abortus*-induced abortion and inflammation of placental tissue[29,30]. Regarding the function performed by the inflammatory response, there is scarce information's concerning the interaction between *B. abortus* and cow placenta. First-ever usage of tested placenta-derived DNA in Iraq was conducted in this study. The current study's results on the retained placenta and their relationship to *Brucella* RBT and PCR status were restricted by the study's small sample size. Although the targeted study design complicates generalization to population-level prevalence, it also may help to explain the study's high PCR positivity rate of 25%.

The PCR analysis only focused on *B. abortus*, resulting in an overall rate of PCR amplification of 11%. 89 % of other PCR samples that did not amplify with the current two targets (*16s rDNA* and *Alk-B* genes) might not identify, potentially leaving other bacteria or *Brucella* species circulating in the targeted population unidentified.

Conclusions

Despite different studies on both the incidence and the seroprevalence of brucellosis in humans and cattle, there are few publications in Iraq on the PCR identification of *Brucella* spp. from cow placentas. In the current investigation, *Brucella* spp. were found in the placentas of seropositive and seronegative cows with a history of abortion

and placenta retention, but animal *Brucella* PCR positivity and RBT positivity were statistically associated.

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Conflict of interest

No conflict of interest was In the current study

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الكشف عن البروسيلا المجهضة في الأبقار التي تعاني من بعض الاضطرابات التناسلية

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تم إجراء هذه الدراسة للكشف عن البروسيلا المجهضة في الأبقار المصابة ببعض الاضطرابات التناسلية.

اختبرت الاضداد وبروتين الدنا في المصل والمشائم للأبقار باستخدام اختبار وردية البنكال وفحص البلمرة المتسلسل حيث تم إجراء اختبارات نسبة الأرجحية واختبار ومربع كاي لتحديد الارتباط الإحصائي بين النتائجبالاعتماد على العينات السريرية اظهرت نتائج فحص البلمرة المتسلسل المستند الى فحص وردية البنكال و ١٦S rDNA ان النسبة الاجمالية بلغت ٣٠٪ (٩٥٪ CI = ٠,١٤٧-٠,٤٩٤) و ١١٪ (٩٥٪ CI = ٠,٠٥٦-٠,٢٨٨) ، على التوالي.ارتبطت النتائج الموجبة لفحص وردية البنكال مع نتائج فحص البلمرة المتسلسل للمشائم و *16s rDNA* وكذلك *AlkB* الموجب على التولي٩٥٪ (OR=٣,٥, ٩٥٪ CI= ١,٢٧٤ - ٩,٤٣٦, χ^2 =٥,٠٢٣,df=١, p =٠,٠٣) و ١٦S rDNA (OR= ٤,٨٥, ٩٥٪ CI =١,٠٢٠ -٢٣,٠٢٨,χ^2 =٤,٧١٤,df=١,p =٠,٠٣)

لم تكن نتائج احتباس المشيمة في الابقار المصابة مرتبطة مع نتائج فحص وردية البنكال او فحص البلمرة المتسلسل (OR=١,٤٠, ٩٥٪ CI= ٠,٢٢٤- ٨,٧٦٨,χ^2 =٠,١٣٠ ,df =١, p =٠,٧) و (OR=٠,٣٣, ٩٥٪ CI =٠,٠٥٧- ١,٨٥٧,χ^2 =١,٧٤١,df=١,p =٠,٢) . بلغت النتائج الموجبة لفحص وردية البنكال في الأبقار ذوات المشيمة المحتبسة ٢٥٪ (٩٥٪ CI = ٠,٠٣٢ - ٠,٦٥١) ، بينما كانت النسبة المئوية للابقار غير محتبسة المشيمة ٣١,٨٪ (٩٥٪ CI = ٠,١٣٩ - ٠,٥٤٩). فضلا عن ذلك سجلت النتائج الموجبة لل ١٦S rDNA وفحص البلمرة المتسلسل المرتبط مع AlkB –PCR في احتباس المشيمة ٢٥٪ (٩٥٪ CI = ٠,٠٣٢–٠,٦٥١) ، مقارنة بالأبقار غير محتبسة المشيمة ، ٩,٨٪ (٩٥٪ CI = ٠,٠٤٦-٠,١٧٨).اظهر تفاعل البلمرة المتسلسل المرتبط مع *16s rDNA* نسبة ٩٪ (٩٥٪CI) في وردية البنكال للابقار المجهضة في حين اظهر نسبة ٢٪(٩٥٪CI) في الابقار المجهضة غير محتبسة المشيمة .