Research Article

Serological, Molecular and Histopathological Study of *Brucella melitensis* Infection in Ewes

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Abstract | Brucellosis in sheep is a highly contagious reproductive infection that may strike almost any breed of sheep and is observed all over the world, causing abortion, infertility, and massive economic losses. **The aims.** This study was to identify brucellosis in ewes and to examine the histopathological changes in the uterine tissue of ewes infected with *Brucella melitensis*. **Methods.** The study were conducted in Kerbala and Babylon provinces from November 2020 to July 2021, using Rose Bengal test (RBT), culture, and polymerase chain reaction (PCR) on blood and uterine tissue samples collected from 139 brucellosis-infected ewes. **Results.** The PCR identified 37 (26.61%) positive cases of brucellosis, which was less than the 41 (29.49%) instances identified by the Rose Bengal test and greater than the percentage identified by the culture, which was 35 (25.1%). In comparison to the results of the culture test, the polymerase chain reaction test was found to be (89.19%) sensitive, (98.04%) specific, and (95.68%) accurate. However, when compared to the results of the RBT test, the sensitivity was (94.59%), the specificity was (94.12%), and the accuracy was (94.24%). Furthermore, the histological changes of the uterus of the infected ewe exhibited extensive histopathological alterations, including necrosis, mononuclear cell infiltrations (MNC), calcification, fibrosis, and severely constricted blood vessels. **Conclusion.** The current study concluded that detecting brucellosis in ewes using a combination of molecular techniques and culture yielded the highest reliable results, suggesting that this method might be employed as a rapid regular screening test.

Keywords | Rose Bengal Test, Polymerase Chain Reaction, molecular techniques, bacterial culturing, Sensitivity, Specificity, *Brucella melitensis,* ewes, Iraq.

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INTRODUCTION

Brucellosis is one of the most important serious worldwide distributed zoonotic diseases that causes severe public health and economic implications (Bundle & McGiven, 2017). Brucellosis in sheep is an infectious reproductive bacterial disease that can affect all breeds of sheep, worldwide distributed and it causes abortion, infertility and enormous economic losses (Radostits et al., 2007; Mahdavi et al., 2018). The transmission of disease occurs between animals via both vertical and horizontal transmissions and the infection can be directly spread from sheep to others or indirectly via infected sheep (Garin et al., 1998; Díaz, 2013). Reproductive failure is considered the principal manifestation of brucellosis in the female such as the birth of an unthrifty newborn or abortion, while in male frequent sterility, epididymitis and orchitis (Radostits et al., 2007).

Abortion due to brucellosis occurs late in gestation and the pregnancy ends before the fetus is born naturally (Vidić et al., 2007, Mats et al., 2020). The fetuses of aborted ovine after experimental and natural infections developed lesions due to systemic infections (Gorham et al., 1986). The uterine tissue of ruminants contains erythritol is a four-carbon sugar utilized by *Brucella* spp. and considered an important factor that may assist the localization and growth of *Brucella* spp and develop an infection in the tissues of the uterus resulting in a large accumulation of bacteria occurs in the placenta, eventually leading to abortion (Poole et al.,1972; Petersen et al., 2013).

Although, the prevalence of brucellosis in Iraq has been reduced by vaccination programs and monitoring control measures, as well as warning farmers of the disease danger and encouraging them to keep their sheep flocks in healthy status, brucellosis is endemic in Iraq and causing infection for many flocks of sheep and causing massive economic losses and threatening the public health status. Therefore, this study aimed to detect *Brucella melitensis* infection in ewes by using serological, molecular, and culturing assays to detect the histopathological changes that appear in the uterine tissues.

MATERIALS AND METHODS

Materials

Brucella agar, nutrient broth, Triple sugar iron agar, Urea agar base, and catalase reagent were provided by (HiMedia Laboratories LLC, United states), gram stain, Ethanol 96%, Hydrogen Peroxide, Urea solution, and oxidase reagent were purchased from (BDH/United kingdom); TBE buffer, Lysozyme, and Ethidium bromide dye were provided from (BIO BASIC INC/United States); Genomic DNA mini Kit (Geneaid, United states); Rose Bengal test (Amcon®), Primers, Polymerase Chain Reaction Premix and water, DNA ladder, and Loading dye were purchased from (Bioneer Korea); Agarose gel (Promega\ United States); disposable Petri dishes (NINGBO EZ MEDICAL INSTRUMENTS CO, China); Disposable syringe, test tubes, and Slides (superstar India), conical flasks (BBL/United states), Sterilized cotton swabs (SterellinLtd., England), micropipettes (Karl Kabl, Germany).

Sample collections

Samples were collected from 139 ewes suspected of brucellosis. They were examined carefully and certain clinical signs were noted. Abortion during late pregnancy was considered the most obvious sign. In addition, fever, depression, loss of weight, giving birth to weak lambs were the determining signs to select the sample from different sites in Karbala and Babylon provinces. Blood samples and ewe's uterine tissues were collected between November 2020 to July 2021. The samples were collected directly from infected ewes by using a sterile equipment like syringe, sterile cotton swabs and screw caps to avoid contamination. Then the samples were transferred to a specialized microbiology lab and were divided into many parts for serological, bacteriological and molecular investigation.

Rose Bengal test and bacteriological culture

The first section was done after using rose Bengal test for the blood samples (Alton et al., 1988). The diagnosis confirmed through the culture and molecular methods, the culture of samples was performed using broth medium and brucella agar. The suspected colonies were stained with gram stain, for microscopic detection of the gram-negative coccobacilli pathogens, after that biochemical tests were used as described in (Alton et al., 1988; Carter & Wise; 2004; Corbel, 2006).

Molecular analysis-DNA extraction and PCR

Molecular technique to confirm the results was done using a PCR test to detect the positive samples according to the manufacture company technique, prepared and directly used 1 ml of freshly evacuated pellets to isolate DNA Extraction and PCR. The polymerase chain reaction test was used as molecular technique to confirm the *Brucella melitensis* infection. The bacterial DNA Extraction Kits were directly used to extract bacterial cells from samples. The present study was conducted by using PCR with a primer of oligonucleotides pair targeting insertion sequence (IS711) 5'AAATCGCGTCCTTGCTGGTCTGA3' and 5'TGCCGATCACTTAAGGGCCTTCAT3', the nucleotide sequence used to detection *B. melitensis* infection in blood and tissue samples and these specific primers and steps were previously described by (Bricker and Halling, 1994).

Histopathological analysis

A specimens from the uterine tissues were collected and fixed in 10% neutral buffered formalin for 24 h , then dehydrated using ascending grades of ethyl alcohol (50-100%) , cleared in xylene (2/changes) , then embedded in melted paraffin wax (60C) , blocked and cut using an ordinary rotary microtome with thickness at 4 µm and the sections were stained with Hematoxylin and Eosin staining. after that, the light microscope was used for the microscopical examination to recognize the histopathological changes (Mohammed., 2021).

Statistical analysis

All variable data was measured by using SPSS version (25) software, according to specificity and sensitivity to the comparison of two techniques, PCR assay was represented as a standard method.

RESULTS

The suspected diagnosis of the ewes infected by *brucella melitensis* primarily depends on clinical examinations and the manifested signs such as abortion during late pregnancy, fever, depression, loss of weight, and giving birth to weak lamb. The techniques used in this study included bacteriological culture, serological test, and molecular method to confirm the diagnosis, the biochemical tests and gram stain were used for more confirmation of the isolates of the suspected *brucella melitensis* colonies and the microscopic examination showed a gram-negative coccobacilli pathogen.

A sharp band of 731 bps was recorded as the positive result by the PCR test as shown in (Figure 1).

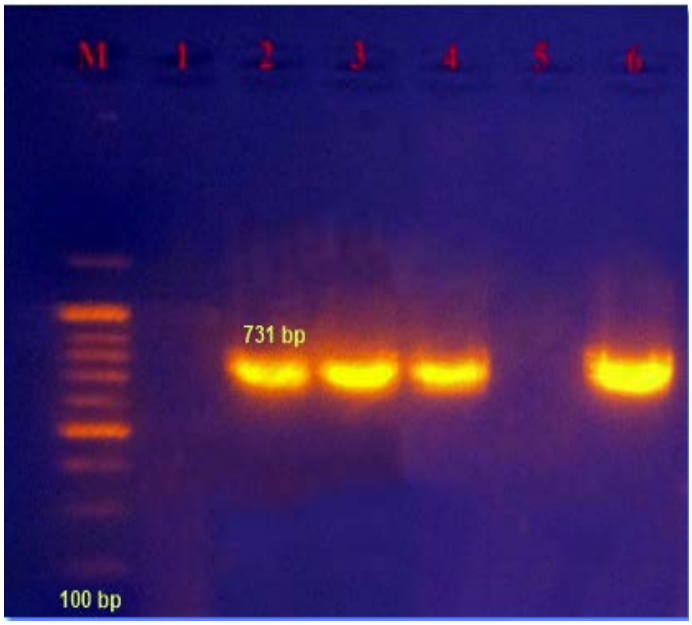


Figure 1: Agarose gel analysis of PCR products, the lane (M) is the DNA ladder; lane 1 is the control negative; lanes numbers 2,3,4 and 6 represent 731 bp for *Brucella melitensis*.

A total of 139 ewes were examined, the result recorded by the PCR was 37(26.61%) positive cases, less than that recorded by the Rose Bengal test, which was 41(29.49%), while the culture showed 35(25.17%) positive cases of brucellosis (Table 1).

The diagnosis that occurred by PCR showed the most reliable and accurate procedure to detect the infection by *Brucella melitensis* in sheep when compared by other tests explaining the specificity and sensitivity of the PCR technique as the standard method compared with RBT and culture, the result found the sensitivity was (89.19%) while the specificity was (98.04%) of PCR with accuracy (95.68%) when compared with the culture results as mentioned in (Table 2), on another side when compared the PCR with RBT results showed that the sensitivity was (94.59%) while the specificity was (94.12%) of PCR with accuracy (94.24%) as mentioned in (Table 3). The prevalence of infection in Karbala province exceeded what was recorded in Babel provinces (Table 4).

Table 1: Shows the percentage of positive cases of infected ewes by *Brucella melitensis* using RBT, Culture and PCR methods

Test	Total examined ewes	Positive NO.	Negative NO.	Percentage %
RBT	139	41	98	29.49%
Culture	139	35	104	25.17%
PCR	139	37	102	26.61%

Table 2: Sensitivity & specificity of PCR compared with culture in diagnosis of *B. Melitensis*

Technique		PCR		Total
		Yes	No	
Culture	Yes	33 *True positive	2 False positive	35
	No	4 False negative	100 **True negative	104
Total		37	102	139

*Sensitivity = [True Positive/ (True Positive + False negative)] χ 100. **Specificity = [True negative/ (True Negative + False positive)] χ 100.

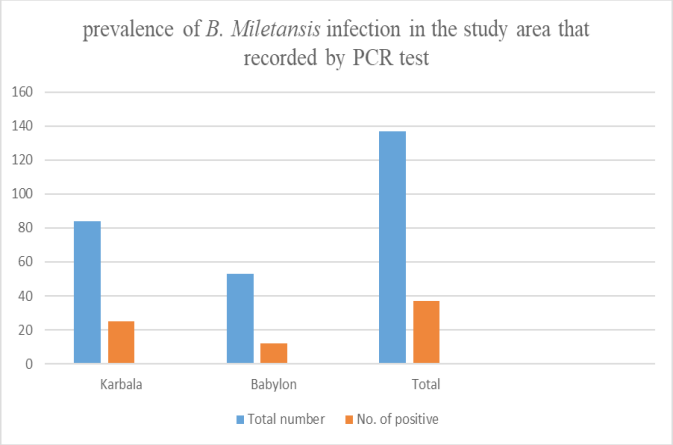
Table 3: Sensitivity & specificity of PCR compared with R.B.T in diagnosis of *B. Melitensis*

Technique		PCR		Total
		Yes	No	
RBT	Yes	35 *True positive	6 False positive	41
	No	2 False-negative	96 **True negative	98
Total		37	102	139

*Sensitivity = [True Positive/ (True Positive + False negative)] χ 100. **Specificity = [True negative/ (True Negative + False positive)]χ 100.

Table 4: Show the prevalence of *B. Melitensis* infection in the study area that recorded by PCR test

Province	Total number	No. of positive	Percentage
Kerbala	84	25	29.76%
Babylon	53	12	22.64%
Total	137	37	27.81%



Brucellosis is known one of the important diseases that cause abortion in infected ewes. The current study was conducted on samples taken from the uterus of infected ewes, which revealed positive cases of *Brucella melitensis* infection. The histological study of infected uterine tissue of ewe with brucellosis using microscopical analysis of the different uterus samples exhibit histopathological changes with different degree of severity were observed, severely congested blood vessels in the subserosal area and vacuolation of uterine muscle cells (Figure 2). Furthermore, infiltration of inflammatory mononuclear cells, fibrosis, and severely congested blood vessels were also evident (Figure 2).

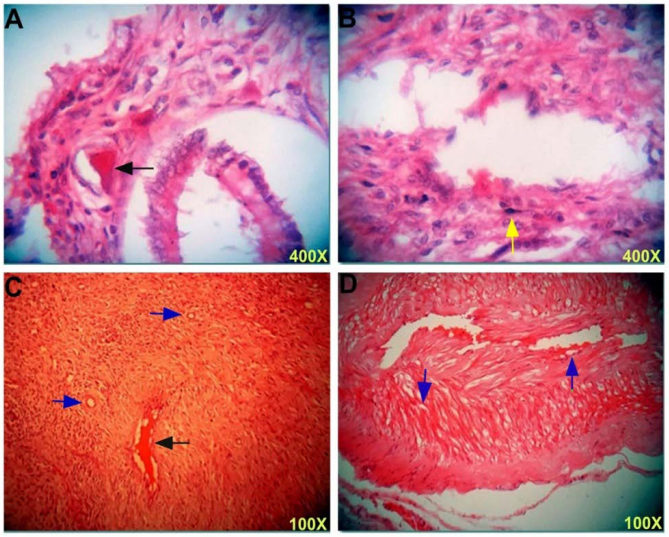


Figure 2: Section in the uterus post infection shows (A) severe congested blood vessels (black arrow) in the subserosal area. (B) Congested blood vessels with few mononuclear cells (yellow arrow) infiltration in the endometrium. (C and D) Congested of blood vessel (black arrow) and vacuolation (blue arrows) of muscle cells. H & E stain, 100X and 400X.

DISCUSSION

Brucella melitensis is one of the serious causes that have massive economic and public health influences as a zoonotic disease that is endemic in many countries including Iraq (Dahl, 2020), which affected mainly sheep as well as humans, the control and eradication of brucellosis in animals based on the most accurate diagnosis of the disease (Corbel, 2006). A total of 139 ewes were examined, the result recorded by the PCR was 37 positive cases, less than that recorded by the Rose Bengal test, which was 41, while the culture showed 35 positive cases of brucellosis that showed different clinical signs as mentioned by (Radostits, et al., 2007). Many diagnostic methods have been improved and developed to detect brucellosis, and latent infection diagnosis are considered one of the problems that exist in endemic areas (Saadat et al., 2017).

Many factors such poor eradication systems of infected animals into the herd, lack hygienic methods such as direct contact with uninfected animals, contaminated food and water, disposal of aborted fetuses and placental membranes as well as un appropriate investigation and prevention programs, all of these factors have affected the spread of brucellosis, control and eradication in animals (Unver et al., 2006; Sadhu et al., 2015, Dadar et al., 2021). The diagnostic tests used for detection Brucella in infected animals may occasionally lack for accuracy (Benkirane et al., 2015).

The present study investigate the prevalence of *Brucella melitensis* infection in ewes in Karbala and Babylon provinces, in Iraq, confirmed by culture and conventional PCR technique as well as a histopathological study to examine the positive cases of infected ewes.

In this study, some cases gave negative results detected by PCR technique, while they gave positive results in the RBT test. The false positive reaction of the serological test may occur due to the cross-reaction with other bacteria (Chenais et al., 2012). This was agreed with Yahaya et al. (2019), who found that RBT has low sensitivity in small ruminants. On the other hand, in this study, some cases gave negative results in the RBT test, while they gave positive results in the polymerase chain reaction (PCR) test. Hence, it was important to use PCR as a technique for routine diagnosis (Marianelli et al., 2008; Junqueira et al., 2013). In a recent study, the presence of *Brucella* DNA was detected in samples collected from animals that gave seronegative results (El-Diasty et al., 2018).

The most reliable definitive diagnosis method is Culture, *Brucella* pathogens were isolated in this study. However, many cases gave negative results in culture, while they gave positive results in serological and molecular tests. The time consumption, the difficulty of performing, culture errors and the procedure, lack sensitivity as well as a high risk of infection when handling culture material to

the operator (Wareth et al., 2014) make the molecular and the serological tests the main methods used for diagnosis of *Brucella* infection in animals and essential for brucellosis investigation. The probability of successfully isolating the pathogen may be affected by contamination, and the isolation rate is low even with experienced laboratories, for that, the negative results of culture cannot make infected with *Brucella* excluded (Bercovich, 1998; Navarro et al., 2004).

The using of conventional tests on serum plays a great role in the screening of brucellosis to detect the infected animals and control the disease (Wareth et al., 2014). The advantage of molecular method for the detection *B. melitensis* is that it saves time and has more accuracy for confirming the diagnosis (Navarro et al., 2004). In addition, the polymerase chain reaction (PCR) technique is widely used as a rapid and sensitive diagnostic method for diagnosing brucellosis and detecting a small amount of DNA in blood samples (Zerva et al., 2001; Kaushik et al., 2006; Ebid et al., 2020).

this study results showed almost the same sensitivity and specificity of PCR of many studies like (Ilhan et al., 2008) who found the sensitivity and specificity of blood PCR 91·1% and 96·5% respectively, and (Gupta et al., 2006) revealed the sensitivity and specificity of PCR were 90 and 100 % respectively, of *B. melitensis* infection in goat.

The histopathological changes were examined in the uterine tissue of infected ewes, and these changes agreed with the histological changes observed by (Ayala et al., 2021) who found the mononuclear inflammatory cells infiltration and aggregation in uterine tissues, along with sites of fibrosis and calcification. a prominent mononuclear cells infiltrate and macrophages surrounded a small focus of calcification in the compact layer of the endometrium. The histopathological changes such as granuloma surrounded by numerous mononuclear inflammatory cells and rimmed by fibrous connective tissue, aggregates of neutrophils, increased interstitial fibrosis, endometrial blood vessels with proliferation of endothelial cells. These changes were observed by (Mansour et al., 2022) in the uterus of sheep infected by *Brucella melitensis*. After infection, female *Brucella* localizes in various lymph nodes of organs such as the internal and external iliac lymph nodes, retropharyngeal, mandibular lymph nodes, supramammary and uterus (Corbel, 2006; Forbes et al., 1996).

CONCLUSION

Brucellosis may pose a real danger due to transmission of infection to other animals and human causing public health risk and economic loss. The study determines the appropriate, highly sensitivity and most accurate diagnostic methods for detecting the disease by using PCR test, in addition to study the histopathological effects of bacteria on infected animals which mainly represented by significant vascular congestion, inflammation and fibrosis.

RECOMMENDATIONS

Molecular tests are important in diagnosing brucellosis infections due to their accuracy and speed in diagnosing infection in addition to traditional tests, as they have high sensitivity and specificity.

Further advance works on the research area of brucella infection in animals in order to reduce the epidemiology of Brucellosis by using vaccines and following eradication programs.

ARTICLE HIGHLIGHTS

Study the percentage of positive cases of infected ewes by *Brucella melitensis* using RBT, Culture and PCR methods. Detection the Sensitivity & specificity of PCR compared with culture and RBT in diagnosis of *B. Melitensis* study the histopathological changes of *Brucella melitensis* Infection in Ewes.

Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The current work has never been published in any language, nor is it being considered for publication in a similar or identical form by any other peer-reviewed journal. The animal experiments were carried out with the approval of the Ethical Committee of the College of Veterinary Medicine.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

FUNDING

This research received no external funding.

NOVELTY STATEMENT

The study investigated Brucellosis in ewes using PCR and examine the histopathological changes in the uterine tissue. We found that combination of molecular techniques and culture provides the highest reliable results , the histological morphology of the uterine tissue revealed sever histopathological alterations, including necrosis, mononuclear cell infiltrations (MNC), calcification, fibrosis, and significant constricted blood vessels.

AUTHOR CONTRIBUTIONS

Ihab G. AL-Shemmari and Ali Hussein Fadhil were concerned with conceptualization, methodology, and formal analysis. Ihab G. AL-Shemmari and Mohammed Assad S. Alkabi were responsible for the investigation, data curation, and study validation; Ihab G. AL-Shemmari, Ali Hussein Fadhil, and Mohammed Assad S. Alkabi were involved in the visualization and original draft preparation; Ihab G. AL-Shemmari and Ali Hussein Fadhil worked on writing review and editing; Ihab G. AL-Shemmari assumed supervisory responsibilities; Ihab G. AL-Shemmari and Mohammed Assad S. Alkabi were followed project administration. Eman Jawad Jabber aided in the histopathological processing and examination. Ihab G. AL-Shemmari provided funding acquisition. All authors approved the final version of the manuscript.

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