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SEROLOGICAL, MOLECULAR AND HISTOPATHOLOGICAL STUDY OF *BRUCELLA MELITENSIS* INFECTION IN EWES

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Objective: The aim of this study, was detection of brucellosis in ewes by using Rose Bengal test (RBT), culture and polymerase chain reaction (PCR) technique as well as investigate histopathological changes in uterine tissue in ewes infected by *Brucella melitensis*.

Methods: the study was conducted by using RBT, culture and PCR on the blood and uterine tissue, samples were taken from 139 of brucellosis suspected ewes, the study begging from November 2020 to July 2021 in Kerbala and Babylon provinces.

Results: Serological, cultural and molecular examinations of samples of suspected infected ewes recorded positive cases with the observation of histopathological changes of the uterus. 139 ewes were examined and show different sings like abortion, fever, depression, loss of weight and ewes that gave birth to weak lambs, 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. The microscopical analysis of uterine samples that collected from infected ewes by *Brucella melitensis* revealed marked histopathological changes. The marked histopathological changes were showed a different severity including infiltrations of mononuclear cell, fibrosis, necrosis, hemorrhage, calcification of tissue and severe congested blood vessels.

Conclusion: This study revealed prevalence of *brucella melitensis* in sheep. The molecular technique and culture showed the most accurate test for detection of brucellosis in animals, *brucella melitensis* infection was detected by using PCR test which considered important to discover the brucellosis infection after use the fast routine screening of herds like RBT. The slaughtered ewes that infected by *brucella melitensis* showed various histopathological changes in the uterine tissue that effect on the health status of the ewe and cause abortion.

Key words: RBT, PCR, molecular tests, histopathological, *Brucella melitensis,* ewes.

Introduction

Brucellosis is one of the most important serious world-wide distributed zoonotic diseases that causes severe public health and economic implications (1,2). *Brucella* spp. are facultative intracellular, Gram-negative bacterial pathogens of several animal species, their principal farm animal hosts are *Brucella abortus* can infect mostly cattle, while *Brucella melitensis* can infect

mostly goats and sheep, as well as *Brucella suis* in pigs and *Brucella ovis* in sheep (3). The transmission of disease occurs between animals via both vertical and horizontal transmissions and the infection can be directly spread from sheep to other or indirectly via infected sheep, their secretions and can infect rams by mating, the infected rams commonly become infected and excrete the pathogen periodically in the semen, cause infertility and abortion in their primary natural hosts (4,5). *Brucella* host specificity has been recorded, *B. melitensis* commonly associated with infection in sheep and goats and observed the most frequently isolated species that causes human cases (2). reproductive failure considered the principal manifestations of brucellosis in the female such as birth of unthrifty newborn or abortion, while in male frequent sterility, epididymitis and orchitis (3).

abortions may caused by infectious or non-infectious factors, infectious agents that cause abortions could be classified as bacterial, fungal, protozoal and viral agents. while the non-infectious factors such as inappropriate nutrition, metabolic disorders, toxemia, stress, hereditary factors, physical factors, etc (6). 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 (3,7). Abortion due to brucellosis occur late in gestation and the pregnancy finished before the fetus is born normally (8). The fetuses of aborted ovine after experimental and natural infections developed lesions due to systemic infections (9).The uterine tissue of ruminants contains erythritol is a four-carbon sugar utilized by *Brucella* spp. and considered an important factor may assist the localization and growth of *Brucella* spp and develop infection in the tissues of the uterus result in a large accumulation of bacteria occurs in the placenta, eventually leading to abortion (10,11).

the *B. melitensis* infection in sheep occurs naturally and causes inflammation of the placenta in pregnant ewes, economic loss may occur in flocks due to the disease by reducing the percentages of lambing, increase abortion and increasing culling rate of rams (2,5). the spread of infectious disease among animals can caused by incomplete vaccination covering programs and animals slaughtering outside slaughterhouses (7). The prevalence of disease in both sheep and humans has been greatly reduced through the use of whole herd vaccination programs, in endemic countries without control programs most of sheep flocks can be infected (2).

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

MATERIALS AND METHODS

Samples were collected from 139 ewes suspected of brucellosis examined carefully and show some clinical signs such as abortion during late pregnancy which considered most obvious sign, fever,

depression, loss of weight and ewes that gave birth to weak lambs, 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 transfers the samples in to the specialized microbiology lab and divided to many parts for serological, bacteriological and molecular investigation.

the study included two sections: The first section was done after using rose Bengal test for the blood samples (2). the diagnosis confirmation occurs through the culture and molecular methods, culture of samples was performed using broth medium and brucella agar, the suspected colonies were stained with gram stain, for microscopic detection the gram negative coccobacilli pathogens, after that biochemical tests were used as described in (2,12,13).

molecular technique, to confirmation the results was done using PCR test to detect the positive samples according to the manufacture company technique, prepared and directly used of 1 ml of freshly evacuated pellets to isolate DNA Extraction and PCR. The polymerase chain reaction test was used as molecular techniques to confirm the *Brucella melitensis* infection. The bacterial DNA Extraction Kits were directly used to extract bacterial cells from samples. The present study was conduct by using PCR with 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 (14).

The second section, the formalin-fixed uterus tissue samples collected for histopathological examination were prepared by using paraffin wax and the sectionesat 4 µm and stained with Eosin and Haematoxylin staining. after that the light microscope used for the slides examination to recognize the histopathological changes (15).

Statistical analysis

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

RESULTS

the suspected diagnosis of the ewes infected by *brucella melitensis* primarily depend on clinical examinations and the signs that manifested like abortion during late pregnancy, fever, depression, loss of weight and ewe that gave birth to weak lamb other sings. The techniques were used in this study which 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 pathogens.

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 compered by other tests explains specificity and sensitivity of PCR technique as 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 other side when compered 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 positive results that recorded by PCR test explained in (Figure 1). In PCR technique, the Extract Master Kit used for Nucleic acids isolation and yielded the expected 731 bp, then use agarose gel electrophoresis to show the isolates.

Table 1: shows the percentage of the 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.

The prevalence of infection in Karbala province exceeded what was recorded in Babel provinces (table 4).

Table 4: Prevalence of *B. Melitensis* in area of study that recorded by PCR test

Province	Total number	No. of positive	Prevalence
Karbala	84	25	29.76%
Babylon	53	12	22.64%
Total	137	37	24.81%

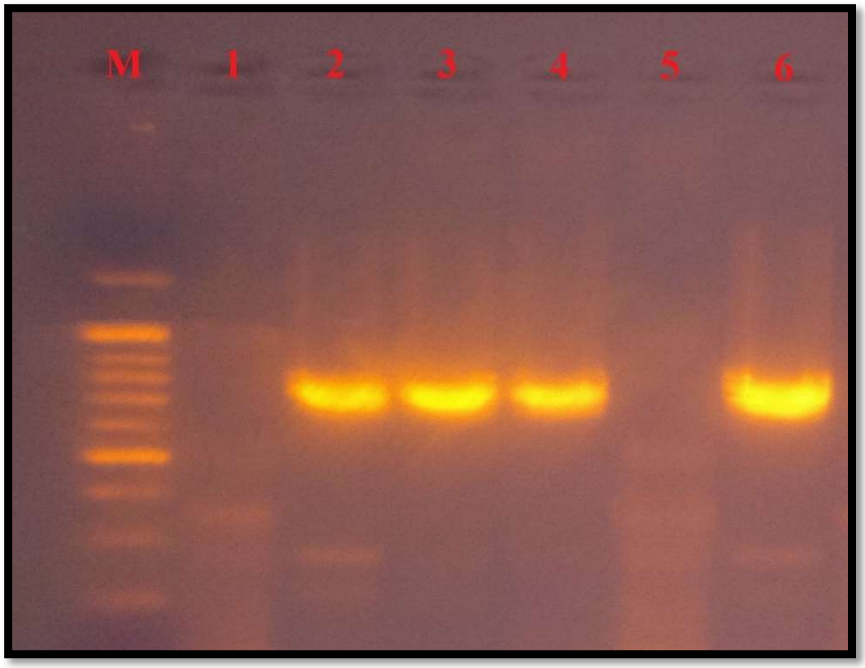

Figure 1: represents a typical result after agarose gel analysis of PCR products, the first lane is the M: DNA ladder, while the lanes numbers 2,3,4 and 6 represent 731 bp for *Brucella melitensis*, lane 5: control negative ddH_2O.

Brucellosis is known one of the important diseases that cause abortion in infected ewes. This has been due to the localization of the infection in placental and uterine tissues. 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 was done by staining performed by the routine procedure using hematoxylin and eosin (H&E) stain, the positive results samples exhibit histopathological changes were noted and have different severity with marked changes have been shown by using microscopical examination analysis of the different uterus samples, Several changes were observed in the uterine tissue such as the infiltration of inflammatory mononuclear cells, fibrosis, necrosis, atrophy, hemorrhage, severe congested blood vessels and calcification of tissue, aggregation of neutrophils and lymphocytes and other inflammatory cells fibrous tissue surrounded the necrotic foci due to the brucellosis effect in the uterine tissue of infected ewes.

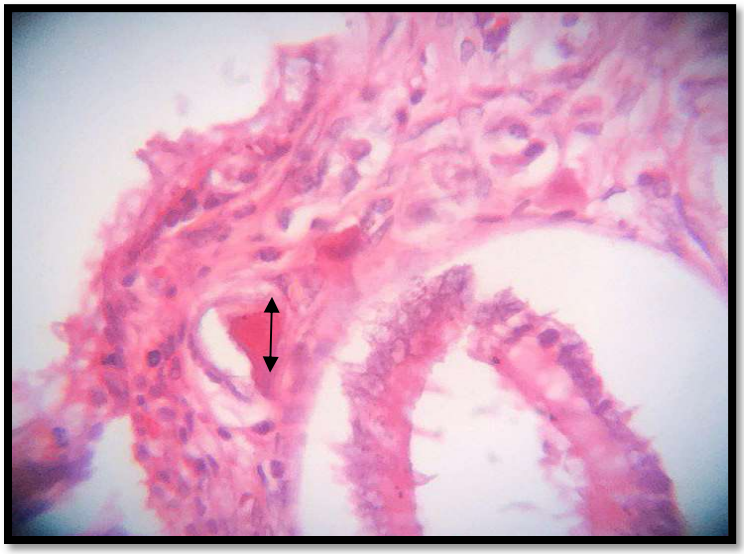

Fig 2: Section in the uterus post infection shows severe congested blood vessels in the subserosal area (H & E stain) 40X.

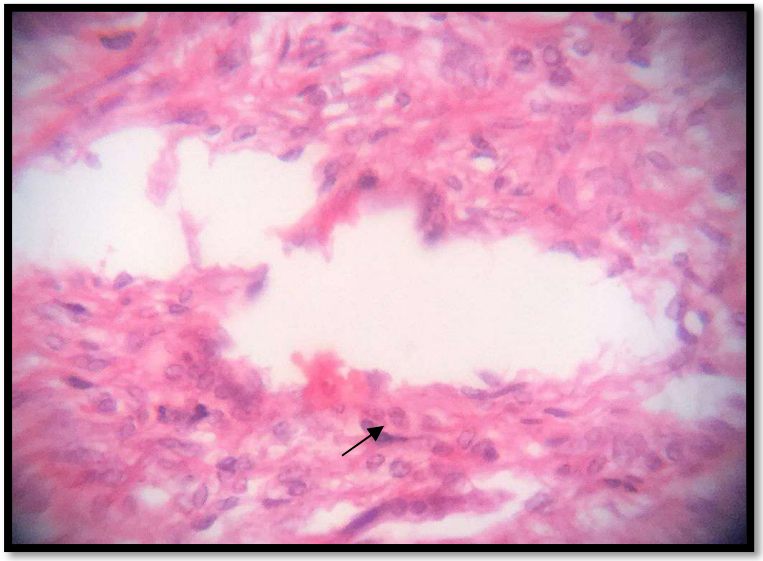

Fig 3: Section in the uterus post infection shows congested blood vessels with few mononuclear cells infiltration in the endometrium (H & E stain) 40X.

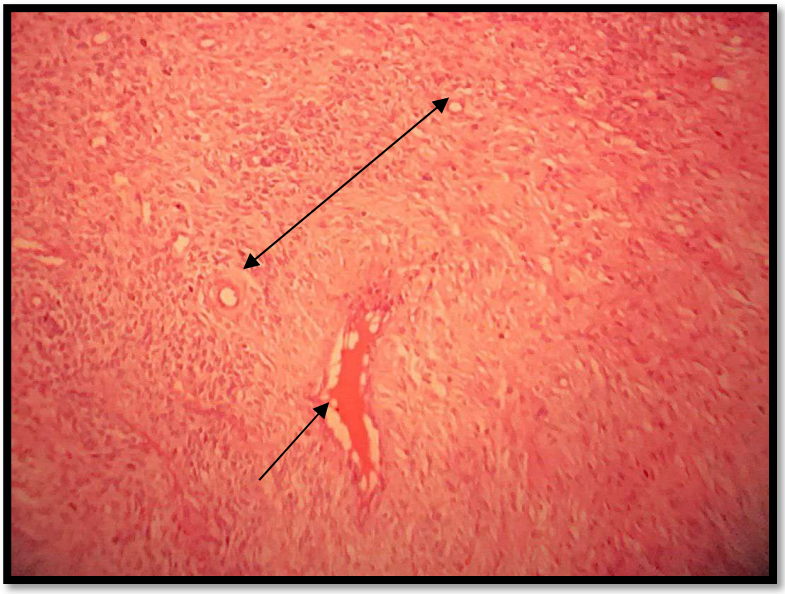

Fig 4: Section in the uterus post infection with *Brucella melitensis* shows congested of blood vessel with vacuolation of muscle cells (H & E stain) 10X.

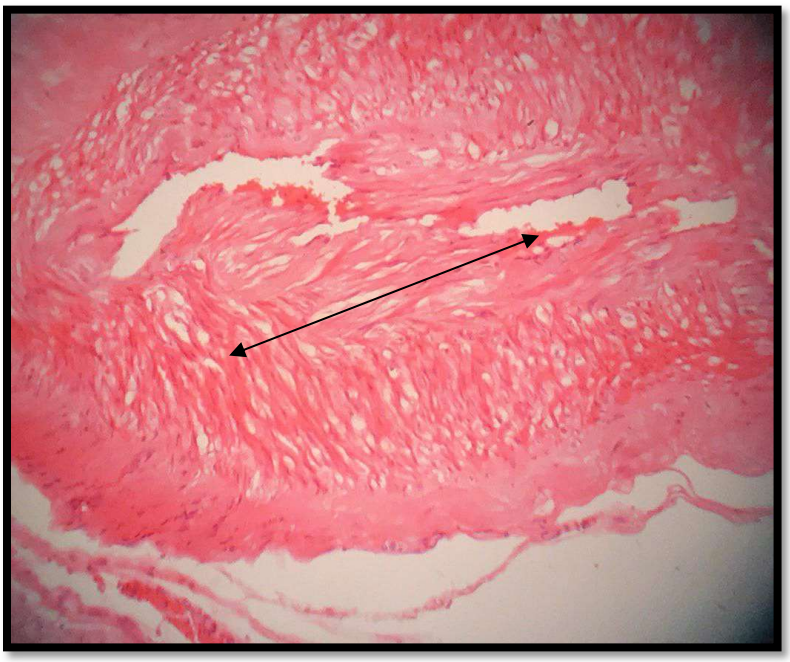

Fig 5: Section in the uterus post infection with *Brucella melitensis* shows vacuolation of muscle cells
(H & E stain) 10X.

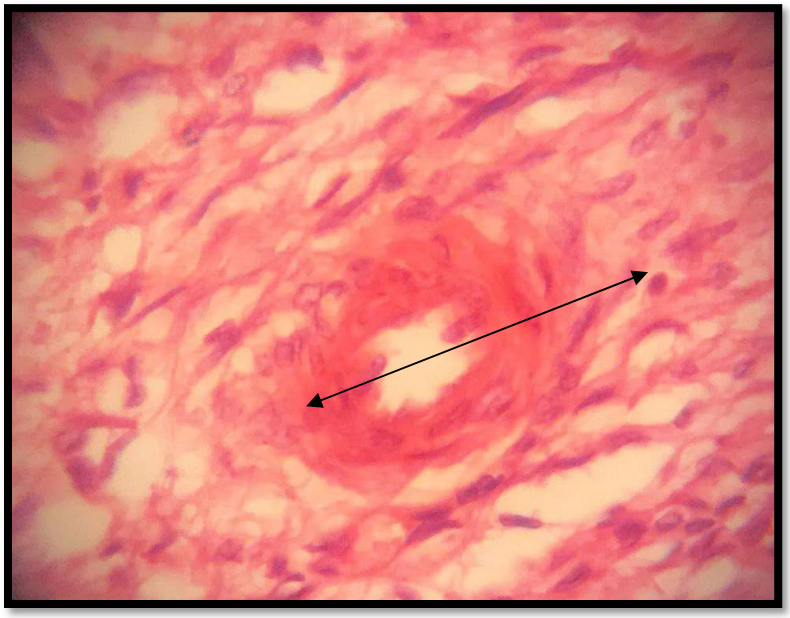

Fig 6: Section in the uterus post infection shows inflammatory cells in dilated congested blood vessels in muscular layer (H & E stain) 40X.

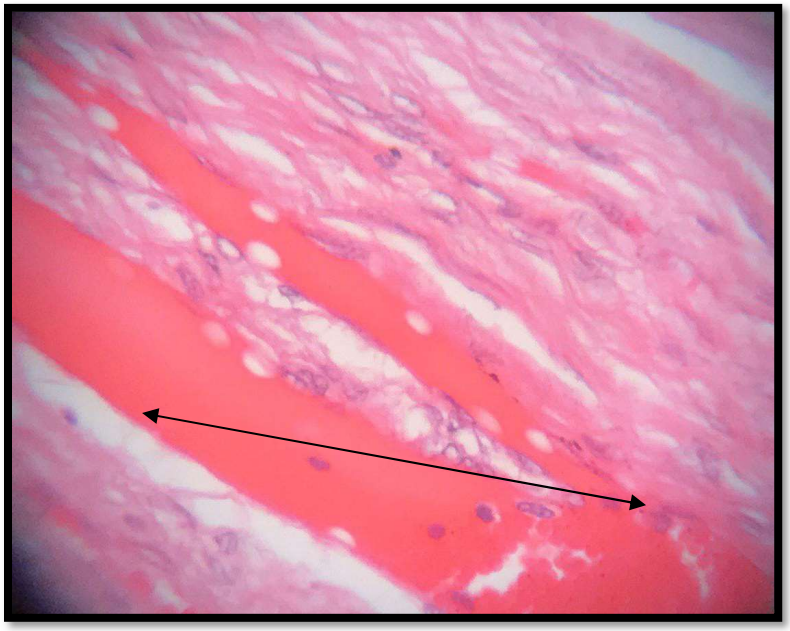

Fig 7: Section in the uterus post infection shows inflammatory cells with severe hemorrhage of blood vessels in muscular layer (H & E stain) 40X.

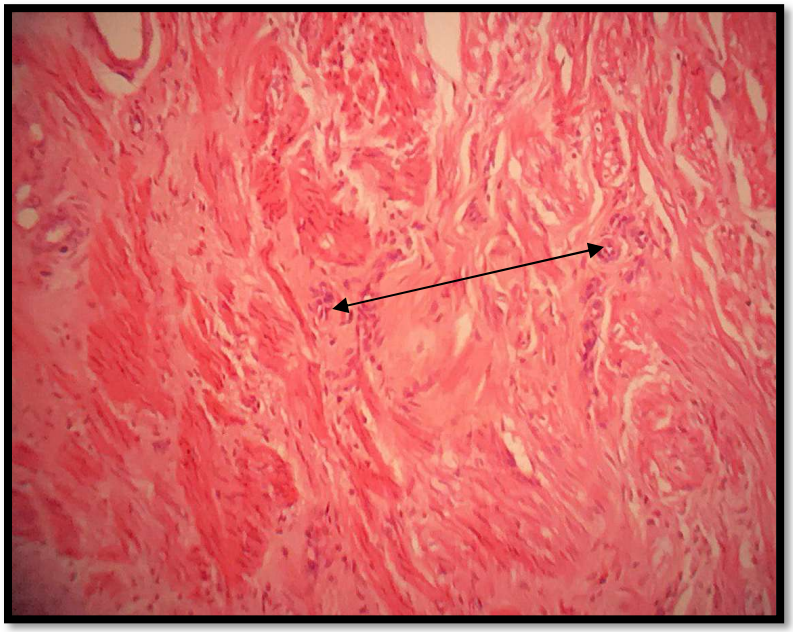

Fig 8: Section in the uterus of post infection shows inflammatory cells particularly mononuclear cells aggregation between muscular (H & E stain 40X)

Discussion

Brucella melitensis is one of the serious causes that have a massive economic and public health influences as a zoonotic disease that endemic in many countries including Iraq, which affected meanly sheep as well as humans, the control and eradication brucellosis in animals based on the most accurate diagnosis of the disease (2). The objective of this study was to determine and detect the prevalence of *Brucella melitensis* infection in ewes and investigate histopathological alteration of uterine tissue; 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 (3). Many diagnostic methods which have been improved and developed to detect brucellosis, and latent infections diagnosis considered one of the problems exist in endemic areas (16). According to the previous publication about brucellosis in Iraq, this disease is endemic and should keep searching to prevent the spread of infections and work to establishing vaccination programs (2).

Many factors such as the entry of infected animals into the herd without quarantine, bad hygiene like contact of uninfected animals with contaminated food and water and disposal of aborted fetuses and placental membranes as well as lack of control and prevention programs all of them have affected prevalence of brucellosis in sheep (17,18). many conditions and factors have made animal brucellosis difficult to control, including increased herd size and trade, control and eradication of brucellosis in sheep need to an appropriate, rapid and accurate method for detect and diagnosis in endemic areas (2). The diagnostic tests used may not detect all infected animals or may appear infection will it not present (19).

This study detect the specificity and sensitivity of PCR technique as standard method compared

with RBT and culture, the result found the sensitivity was (83.78%) while the specificity was (98.04%%) of PCR with accuracy (94.24%) when compared with the culture results, on other side when compered the PCR with RBT results showed that the sensitivity was (89.19%) while the specificity was (98.04%) of PCR with accuracy (95.68%), this study results showed almost the same sensitivity and specificity of PCR of many studies like (20) who found the sensitivity and specificity of blood PCR 91·1% and 96·5% for respectively, and (21) revealed the sensitivity and specificity of PCR were 90 and 100 % respectively, of *B. melitensis* infection in goat.

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 of culture material to the operator (22). 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 isolation successful markedly reduced or it makes it effected by contamination as well as the rate of isolation is low even with experienced laboratories, for that the negative results of culture cannot make infection with *Brucella* excluded (23,24).

The using of conventional tests on serum play a great role for screening of brucellosis in detect the infected animals and control programs of the disease (22). the effective protocol for control of brucellosis by the quick detection of *B. melitensis* by using PCR one of the most accurate technique and has sensitivity more than traditional methods, the advantage of this method for detection *B. melitensis* saves time and it has more accuracy for confirmation the diagnosis (24).

development of the molecular test is the big step toward the improvement methods of diagnosis for detection many infections and *B. melitensis* infection one of the important zoonotic diseases need that fast technique to prevention and control of the infection with vaccines, PCR technique are widely used for the rapid diagnosis of brucellosis and detects small quantity of DNA in blood samples (25,26). Therefore, PCR technique considered the more rapid and sensitive diagnostic method for brucellosis (27).

In this study, some cases gave negative results in PCR technique, while they gave positive results in RBT test. The confirmation of brucellosis occurred by using PCR in blood samples that were gave positive results by RBT, the false positive reaction of serological test may occur due to the cross reaction to other bacteria (28), the brucellosis diagnosis by using PCR for blood samples is applicable (29). While RBT has low sensitivity in small ruminants (30). 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. Diagnosis should be accomplished by molecular diagnosis, detect *Brucella* DNA can be done by using PCR assay in seronegative animals for that it was important to use PCR as a technique for routine diagnosis (31, 32). In a recent study, the presence of *Brucella* DNA was detected in samples collected from animals gave seronegative results (33). the PCR kit used for the detection of the *Brucella* in the test samples are easily obtained from animals for DNA extraction such as blood and milk samples and other samples include tissues, serum, body fluids and semen. direct test can be attempt to the primary cultures of *Brucella*.(34).

The present study revealed the prevalence of *Brucella melitensis* infection in ewes in Karbala and Babylon provinces, in Iraq; positive cases of serological were confirmed by culture and conventional PCR technique additional histopathological study to notice the pathological alterations in uterine tissue samples of infected ewes.

The histopathological changes were examined of uterine tissue of infected ewes and These changes agreed with the histological changes observed by (35) 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 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 (36) in uterus of sheep infected by *Brucella melitensis*. After infection, in female *Brucella* localizes in various lymph nodes of organs of such as internal and external iliac lymph nodes, retropharyngeal, mandibular lymph nodes, supramammary and uterus (2,37).

Conclusion

This study revealed prevalence of *B. melitensis* in sheep. The serological, molecular tests and culture help to detect the *Brucella* infection and they confirm a suspected diagnosis arising from clinical signs and paved the way to study and notice the histopathological changes in uterine tissue that occur due to infection of sheep with *Brucella melitensis.*

The molecular technique showed the high accuracy test for detection of brucellosis in animals, *B. melitensis* infection was detected by using PCR test which considered important to detect the brucellosis infection after use the fast and routine screening of herds like RBT as well as culture.

The slaughtered ewes that infected by *B. melitensis* showed various histopathological changes in the uterine tissue that effect on the health status of the ewe and cause abortion. Brucellosis may pose a real danger due to transmission of infection to other animals and humans causing public health risk and economic loss. Therefore, we need to continue researching and diagnosing positive cases, and the veterinary officials in Iraq must keep efforts to fight to improve their diagnostic methods and vaccination programs in order to decrease or eradicate this important zoonotic disease.

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