



Pathological and molecular diagnosis of *Brucella melitensis* in the fetal and placental tissues of aborted ewes in Al-Najaf city

Hameed AbdulHussan AL-tememy* Kahalil Hassan Al-jubort**
Ban Abbass Abdulmajeed ***

*College of Veterinary Medicine, University Baghdad

**College of Veterinary Medicine, University Baghdad

*** College of Medicine, University Baghdad

E-mail: hahahatmtmtm@yahoo.com, Mobile: 07801579878

Abstract:

Brucellosis is one of the common bacterial zoonosis in the worldwide caused by organisms belong to the genus *Brucella*, However, animal Brucellosis is a serious problem worldwide and is endemic globally and disease of sexually matured animals and commonly transmitted to other animals by direct or indirect contact with infected animals or discharges such as: aborted fetuses, placental membranes or fluids. Fifty samples from internal organ of aborted fetus and the same number of aborted placenta were collected from aborted ewes positive to Rose Bengal test. For isolation and identification of *Brucella* from placenta and stomach contents, the standard procedures (Alton *et al.*, 1988) were followed. The isolates were further confirmed by molecular techniques. The results showed that *Br. melitensis* was isolated from all animals that expressed Rose Bengal and serum agglutination test positive. The agglutination with monospecific A and M antisera were performed and these test revealed that the bacterial isolates expressed three Biovar as following, Biovar 1(M,50%), Biovar 2 (A,26%) and Biovar 3 (0,24%). The number of this Biovar were varied according to collect area of the samples, high percentage (44%) was seen in the ALmanitherh, followed by AL Shabeka (30%) and AL Kuzweenah (26%).

The results of PCR assay explained that 22 bacterial isolates showed a single amplified DNA product (44%), two bands at the level of 273 and 680 bp were seen in 8 isolates (16%) and four bands at 273,680,750 and 850 bp were reported in 20 isolates(40%).

Key words : *Brucella melitensis* - sheep - Iraq- PCR.

دراسة مرضية وتشخيص جزيئي لجرثومة البروسلا مليتينسس المعزولة من أجنة ومشيمة الأغنام
المجهضة في محافظة النجف الاشرف. العراق

حميد عبد الحسين التميمي* خليل حسن زناد**
بان عباس عبد المجيد ***

*كلية الطب البيطري، جامعة بغداد

**كلية الطب البيطري، جامعة بغداد

كلية الطب، جامعة بغداد

الخلاصة:

يشكل مرض الإجهاض الساري مشكلة كبيرة للإنسان والحيوان في العالم عموماً وفي العراق خصوصاً، لتسببه في حالات إجهاض كثيرة عند الإناث. ونظراً لوبائية المرض وما يتسبب عنها من أضرار صحية واقتصادية جاءت فكرة دراستها في هذه الأطروحة والتي قامت على جمع 100 عينة توزعت على 50 مشيمة وجنين مجهض و50 مشيمة سليمة وقد تناولت الجوانب الآتية: عزل وتشخيص المسبب ومعرفة الأنواع الخاصة به باستعمال الأجسام المضادة الأحادية (Mono colonial antibody) وكذلك دراسة الحامض النووي للمسبب عن طريق تقنية (PCR) باستعمال بادئات (primers) خاصة للحامض النووي للمسبب. وقد تمخضت الدراسة عن النتائج الآتية:

- أن العنبر الرئيسية المسببة للمرض والأكثر شيوعاً كانت حسب النسب الآتية: بروسلا ملتينسيس نوع 1 (biover) بنسبة 50%، بروسلا ملتينسيس نوع 2 بنسبة 26%، بروسلا ملتينسيس نوع 3 بنسبة 24%.
- أن استخدام فحص PCR في تشخيص مرض البروسلا يكون أكثر ائناً ودقاً وأقل وقتاً.
- من خلال تقنية تفاعل (PCR) للحامض النووي للمسبب تبين وجود حزمة (bind) إضافية فضلاً عن الحزمة الرئيسية عن طريق الوزن الجزيئي (bp) أنفاً، ومن معرفة الوزن الجزيئي للحامض النووي لعنبر اللقاح، اتضح أن الأغنام المجهضة تحمل حزمة اللقاح فضلاً عن الحزمة الخاصة بالمسبب المرضي المعزول وعليه يجب أن تكون عترة اللقاح مطابقة للعترة المسببة للإجهاض نفسها المعزولة في الحقل.

Introduction:

Brucellosis is a major emerging zoonotic caused by the small, non-motile gram-negative and intracellular coccobacilli belonged to the genus *Brucella* (1). This disease induces important economic loss in the farm animals through abortion, infertility, birth of weak and dead offspring, placental retention and reduction of milk yield (2).

Brucellosis is mainly a disease of sexually matured animals and commonly transmitted to other animals by direct or indirect contact with infected animals or discharges such as: aborted fetuses, placental membranes or fluids. Infection to human results from direct contact with infected animals and consumption of contaminated milk and its products (3).

However, the main causes of ovine and caprine brucellosis is *B. melitensis* (4) which induced abortion at the last two months of gestation in addition to orchitis and epididymitis (5). Although brucellosis in domestic animals has been eradicated in great number of European countries, in some Mediterranean and middle eastern countries but the risk of reintroduction of the disease still exists through spill-over from wildlife that are considered to be natural reservoirs (6,7).

Reliable and sensitive diagnostic tools play a crucial role in the control of brucellosis in livestock, wildlife and

humans. Currently, diagnosis of brucellosis is mainly performed by blood and tissue cultures and classical serological tests such as: Rose Bengal Plate Test (RBT), Tube Agglutination Test (TAT) and Slow Agglutination Test (SAT). However, these tests show low sensitivity due to produce cross reactions with other Gram negative bacteria having antigenic similarities with *Brucella* (8).

The sensitivity of the bacteriological culture methods depends on the viability and numbers of *Brucella* in the sample, and the nature of the sample which is commonly contaminated with other bacteria. Thus, culture methods are not always successful as they are time-consuming and the handling of microorganism is hazardous (9).

Antibody response against brucellosis is dependent upon stage of infection of sample collection in which are not recorded in the first 12-16 days post infection in goat experimentally infection with *B. abortus* 19, also these Abs are not detected in the chronic brucellosis (10), therefore false-negative results have been noticed in diagnosis of brucellosis, (11).

Molecular diagnostic techniques represent an important breakthrough in the diagnostic practice. A number of genus- or species-specific conventional PCR assays using primers derived from different gene

sequences from the *Brucella* genome, such as 16S rRNA(12).

These assays were adapted for application to *Brucella* detection in different clinical specimens. In the majority of studies, conventional PCR proved to be a good mean to detect *Brucella* DNA from clinical specimens such as different samples from goats (13) and bovine, while, the brucellosis of the sheep has received comparatively little attention by using milk as material.

Brucellosis is endemic in Iraq and large number of aborted cases occur every year with stillbirths without accurate diagnosis of the etiology. The purpose of this study was to investigate the efficiency of the PCR assay as a diagnostic tool for the detection of *B. melitensis* DNA in the stomach content of fetus after abortion and to compare its results with traditional bacteriological culture methods.

Materials and methods:

Culture media

Tryptic soya broth and Farrells medium as selective media which were prepared by addition of the following antibiotics quantities per each liter of basal medium(TSA): bacitracin (25mg), polymyxin B sulphate (5mg), nalidixic acid (5mg), nystatin (100,000 units), vancomycin (20mg), cycloheximide (50mg).

Sample collection

1-Blood samples

We collected 5ml of the blood aseptically from jugular veins of 50 aborted ewes during the course of the study from March to June 2011. These samples were used to Rose Bengal test and tube agglutination test.

2-fetal and placental samples

We collected 50 aborted fetal samples and 50 placental samples from aborted ewes which showed positive Rose Bengal test. Each sample was divided into two parts, one part was used for

histopathological study, the second part was used for bacterial isolation.

Histopathological technique.

Small pieces from internal organs of the fetus which included liver, spleen, lung and kidney and placenta were fixed in 10% neutral buffer formalin for 72 hrs then we used the routine histological processing to prepare the sections according to (14).

Bacterial isolates:

Abomasum contents were removed by searing an area of the stomach wall with a heated spatula, plunging the tip of a sterile injector through the seared area and by cotton swab transferring some of the contents and inoculated to tryptic soya broth and incubated at 37°C for 2-3 days. Frequently, field samples are contaminated with other bacteria, thus, selective media should be used to avoid overgrowth by fast growing agents. The most widely selective media used are the Farrell's, medium which is prepared by addition of the following antibiotics and quantities per liter of basal medium(TSA): bacitracin (25mg), polymyxin B sulphate (5mg), nalidixic acid (5mg), nystatin (100,000 units), vancomycin (20mg), cycloheximide (50mg). medium.

After 48-72h of incubation at 37°C, *Brucella* colonies are 0.5 to 1.0 mm in diameter with a convex and circular outline. Smooth strains are transparent and pale yellow, resembling droplets of honey with a shiny surface where observed in transmitted light. Rough colonies are more opaque with a granular surface. Unique suspected colony removed and resuspension on new plate until pure culture received.

Preparation of material for nucleic acid extraction from colony

Suspected colonies from TSA plates were streaked on TSA and incubated at 37°C for 4 to 5 days. After visible growth on the plate, colonies were washed with 2 to 4 ml of PBS (pH 6.4).

The suspension in PBS was diluted according to MCferling tube up to $1+10^9$.

The bacterial culture dilution was transferred to 1.5 ependdorff tube and then centrifuged for 1 minute at 14000 rpm . The supernatant was discarded and the pellet was used for extraction of nucleic acid. Two hundred μ l of GT Buffer were added to the tube and the cell pellet was resuspended by shaking vigorously and incubate at room temperature for 5 minutes . (Genomic DNA mini kit Blood/Cultured).

- Two hundred μ l of absolute ethanol were then added to the sample lysate and immediately mixed by shaking vigorously .When a precipitate appeared , it was broken up by pipeting.
- A GD column was placed in a 2 ml collection tube .
- All the mixture (including any precipitate)was transferred to the GD column and centrifuged at 14000 rpm for 2 minutes .
- The 2ml collection tube containing the flow through was discard and the GD column was placed in a new 2 ml collection tube.
- WI buffer was added in 400 μ l of to the GD column and centrifuge at 14000 rpm for 1 minute .
- The flow –through was discarded and the GD column was transferred to a 2 ml collection tube.
- Six hundreds μ l of wash buffer(ethanol added) was added to the column and centrifuge at 14000 rpm for 1 minute .

- The flow –through was discarded and the GD column was transferred to a 2 ml collection tube and centrifuge again for 3 minutes at 14000 rpm to dry the column matrix.

- One hundred μ l of preheating elution buffer (or TE) were added to the center of the column matrix and let it stand for 3-5 minutes or until the Elution Buffer or TE was absorbed by the matrix.

- Centrifugation was then done at 14000 rpm for 1 minute to elution the purified DNA.

Quantitation and quality assessment of DNA

Method:

Quality and purity of DNA were checked by submarine agarose gel electrophoresis using 0.8% agarose in 0.5X TBE (pH 8.0) buffer . Ethidium bromide (1%) was added in a volume of 5 μ l/100ml. The wells were loaded with 5 μ l of DNA preparations mixed with 1 μ l of 6X gel loading buffer dye. Electrophoresis was carried out at 5V/cm (75 Volte)for 40 min at room temperature and then the DNA was visualized under UV transilluminator.

PCR:

Primers: two pairs of primers (synthesized by Alpha DNA Monteral) were used for PCR amplification as per the details given in Table (1).all selected primer sequences were then checked for possible cross-hybridization using the BLAST,(BLAST,2007).

Table (1) List of Primers

References	Product length (bp)	Sequence 5'– 3'	Name of primers
BLAST 2007	482	TG CCG ATC ACT TAA GGG CCT	(F)
		GCG CTG ACC AAG GGC CAA AA	(R)

(F) = Forward primer; (R) = Reverse primer

b) **Method** :PCR of suspected colonies was carried out in final reaction volume of 25 μ l in thermal cycler (MyCycler, Bio-Rad, USA). Quantity and concentration of various components used for colony PCR are shown in tables (2) Steps and conditions of thermal cycling for two primer pairs in PCR are shown in Table (3).

Table (2) Quantity and concentration of various components used in PCR

1	2+KAPA2GRobust hotstart Ready Mix contains 2mM Mgcl ₂ ,at 1X	12.5 ul
2	Forward Primer (10 uM)	2 ul
3	Reverse Primer (10 uM)	2 ul
4	Template DNA	3 ul
5	PCR Grade water	5.5 ul

Table (3) Steps and conditions of thermal cycling for different primer pairs in PCR

Cycling conditions				
Initial denaturation	Denaturation	Annealing	Extension	Final extension
95 10 min	95°C 1 min	63C 1min	72 C 1 min	72 C 7min
1	Repeated for 35 cycles			1

Visualization of PCR products by agarose gel electrophoresis

Ladder ; The KAPA Universal Kit is designed for determining the approximate size and quantity of double-stranded DNA on agarose gel. KAPA Universal Ladder Kit contains eighteen DNA fragments (in base pairs): 100,150,200,300,400,**500**,600,800,**1000**,1200,**1600**,2000,3000,**4000**,5000,6000,8000, and 10000,The KAPA Universal Ladder contains four reference bands (500,1000,1600,and 4000) for orientation .Kits are formulated with DNA loading dye for direct loading on agarose gel.

Method;-To confirm the targeted PCR amplification, 10 µl of the PCR products from each tube was mixed with 3 µl of 6X gel loading buffer and electrophoresed along with DNA molecular weight marker on 1.0% agarose gel containing ethidium bromide (0.5 µg/ml) at constant 75V for 75 min in 0.5 X TBE buffer. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel

documentation system (SynGene, Gene Genius Bio Imaging System, UK).

Results:

Isolation and identification,
The results showed that round, glistening, smooth or mucoid colonies on plates of agar plates and TSA at 48 hrs post incubation at 37C°.The colonies were streaked on blood agar and MacConky agar plates (Fig:2), in addition to non-hemolytic isolates on blood agar as well as non-lactose fermenting were seen.

The isolates were streaked on blood agar (BA)and MacConkey agar (MA) plates. The non-hemolytic isolates on BA as well as non-lactose fermenting isolates on MA were preliminary presumed to be of *Brucella* Species identification and biotyping. The culture smears showed Gram-negative coccobacilli in Gram's staining . The colonies were round, convex, smooth margin, translucent, honey-colored, glistening , and bluish on *Brucella* selective media. There was no agglutination with Acriflavine, The

cultures were positive for biochemical reactions (catalase , oxidase, nitrate reduction, and urease tests). There were some variations in urease activities shown between reference strains, rapid, slow, and moderate , respectively .

The colony morphology of all 50 isolates was smooth and all were negative in the agglutination test with Acriflavine and positive to oxidase and urease.. After agglutination by polyclonal antiserum, all of the 50 *Brucella* isolates were identified as *B. melitensis*.

Table(4) showed the results of biochemical test of bacterial isolates

Name the test	The result
Acriflavine	+ ve
Oxidase	+ ve
Catalase	+ ve
Urease	+ ve

According to the morphology of bacterial colonies ,gram stain and biochemical test, the result reveled that, these isolates of *B. melitensis* were coincidence with PCR results.

We recorded that all collecting samples from aborted ewe that showed Rose Bengal test positive expressed positive *Brucella* isolates and these bacterial isolates was variable according to area of sample collecting (Table:5).high bacterial isolates were recorded in the AL.manathera (44%) followed by Al shabaka(30%) and kuzweenah(26%) these result may be due to variable in the vaccination protocol or due to environment condition that favor *Brucella* infection,

Biotyping

25 out of 50 *Brucella melitensis* isolates, were *B. melitensis* biotype 1 , , (50%), and 13 was *B. melitensis* biotype 2 (26%),12 were identified as *B. melitensis* biotype 3 (24%) after testing positive with mono specific anti A and anti M sera table(5).

Table: (5) showed biovar of the isolated *B.melitensis*

NO.of samples	Area of collection	+ev <i>Brucella</i> isolates	Brucella biotype			%
			M	A	O	
1-22	Al manathera	22	12	4	6	
23-35	Al kuzweenah	13	8	3	2	
36-50	Al shabaka	15	5	6	4	
		50	%50	%26	%24	%

The current study revealed that *B. melitensis* biotype 1 is the main biotype in the AL-Najaf city followed by biotype 2 while biotype 3 is the less one .

Confirmation by PCR ; In the present study ,PCR assay was used for diagnosis of *B.melitensis* from pure culture, we demonstrated that all culturing bacterial isolates showed positive PCR assay fig(1).

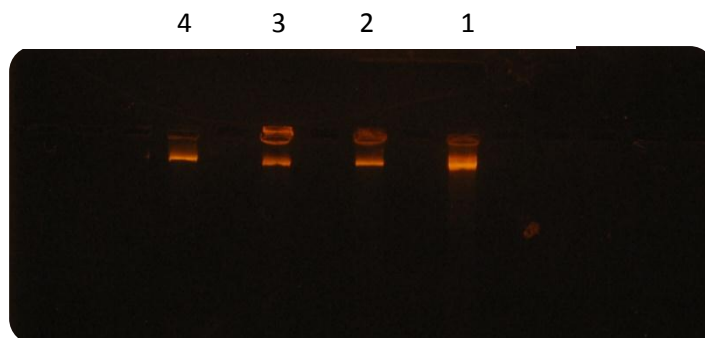


FIG (1): Ethidium bromide stained agarose gel of *Brucella melitensis* DNA extraction from culture of stomach content of aborted sheep fetuses.

The Result of amplification of *Brucella Melitensis* gene by PCR

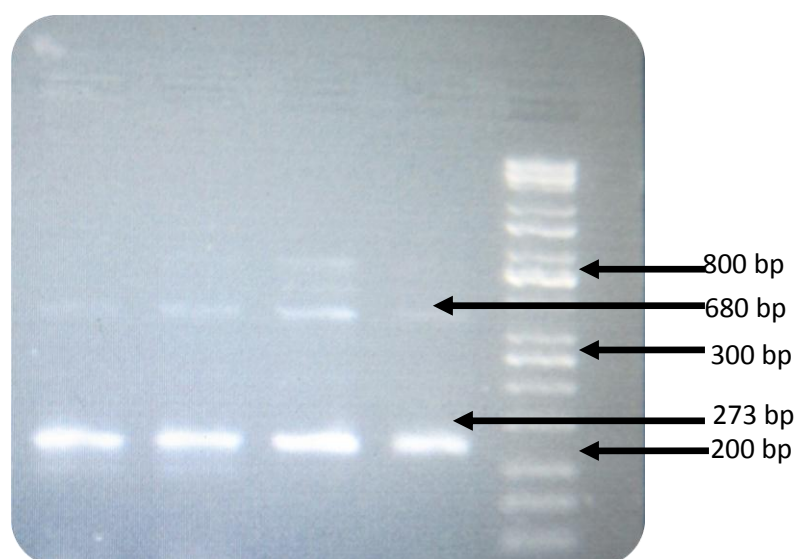


Figure (2) shows the amplification of *Brucella melitensis* gene. Line 2,4 and5 showed 2 band at 273,680 bp, Line 3 also showed 4 band at 273,680 ,750 and 850 bp,

Histopathology

Liver; Histopathological section in the liver of aborted fetus shows discrete hepatocytes, fibrin deposition mononuclear cells infiltration, in addition to mononuclear cells infiltration in the portal area and vacuolar degeneration of hepatocytes fig(3,4).

Placenta; Histopathological section in the placenta of aborted sheep showed necrosis and sloughing of trophoblastic cells of the chorionic villi with neutrophil infiltration in the villous stroma(Fig:7) and intervillous space as well as in necrotic area .in other animals ,the placenta revealed necrotic the chorionic plate,in addition large multiple area of

dystrophic calcification .The decidual area expressed congestion of the blood vessels with neutrophils in their lumen and necrosis of extratrophoblastic cells and neutrophils infiltration in the necrotic area (Fig:7)

The fetus;We recorded necrosis of the hepatocytes with fibrin networks and neutrophils in the dilated sinusoids (Fig:3),in other section severe fibrin networks deposition in the liver parenchyma as well as aggregation of mononuclear cells scattered through liver parenchyma (Fig:4).

The lung of aborted fetus expressed congestion of the blood vessels with neutrophils in their lumen and in the

alveolar space (Fig:5). In other case, mononuclear cells aggregation in the wall of the bronchi were seen and severe hemorrhage and neutrophils infiltration in the alveolar space .

Microscopic section of the kidney revealed necrosis of renal tubules with fibrin deposition in the interstitial tissues(Fig:6) as well as necrosis of epithelial lining cells of dilated renal tubules(Fig:6).

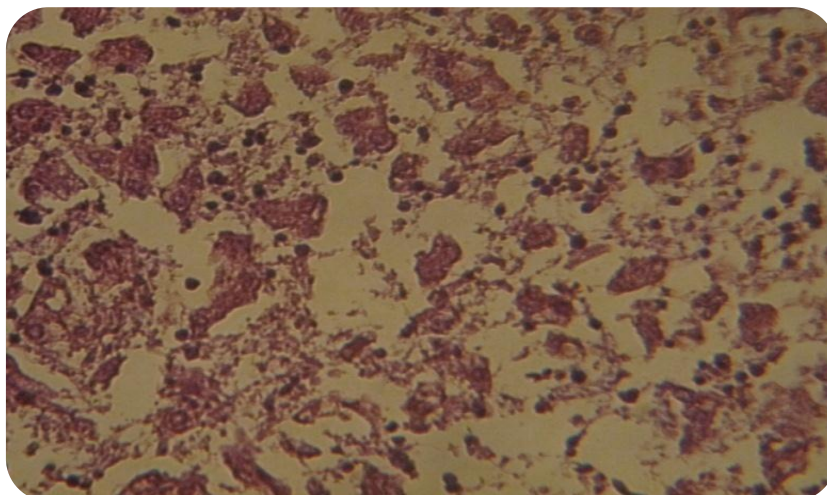
Lungs:The lung of aborted fetus shows, fibrin network deposition and inflammatory cells infiltration in the interstitial tissues (Fig 5), the interlobular septa and the alveolar space with mononuclear cells infiltration, in

other fetus ,we reported fibrin network in the alveolar space with mononuclear cells in the inter alveolar septa.

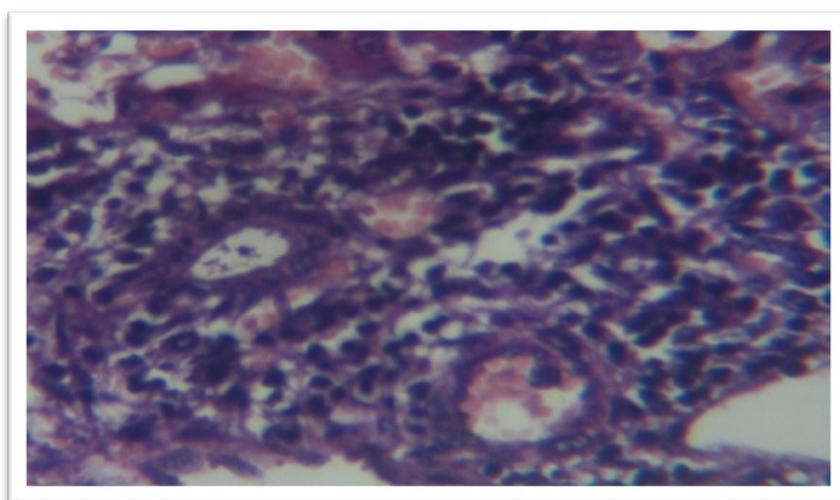
Kidney:Histopathological section in the kidney of aborted fetus shows necrosis of renal tubules with fibrin deposition in the interstitial tissue (fig:6).

Spleen: the spleen showed marked depletion of white pulp with fibrin deposition and inflammatory cells in red pulp .

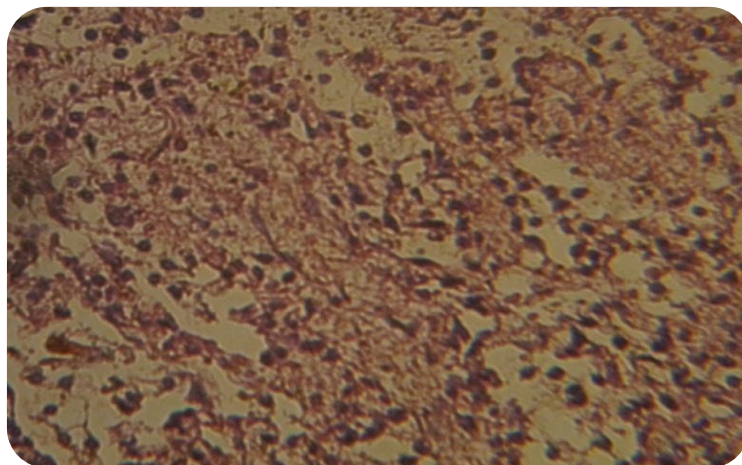
Placenta; The microscopic section revealed calcium deposition in the chorionic plates with congestion of blood vessels and neutrophils in their lumen (Fig7).



Fig(3). Histopathological section the liver of aborted fetus showed discrete hepatocytes with inflammatory cells in dilated of sinusoids(H&E stain 400X) .



Fig(4). Histological section in the liver of aborted foetus shows mononuclear cells aggregation in the portal area and congested dilated sinusoids(H&E stain, 400X)



Fig(5). Histological section in the lung of aborted fetus showed fibrin networks deposition with inflammatory cells infiltration (H&E stain, 400X)

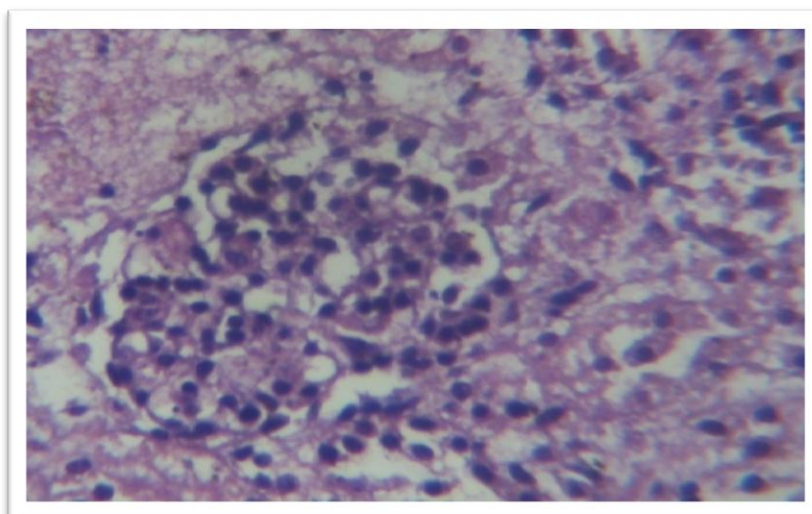


Fig:6. Histological section in the kidney of the aborted fetus showed necrosis of renal tubules, fibrin networks deposition with inflammatory cells infiltration in the interstitial tissues(H&E stain ,400X)

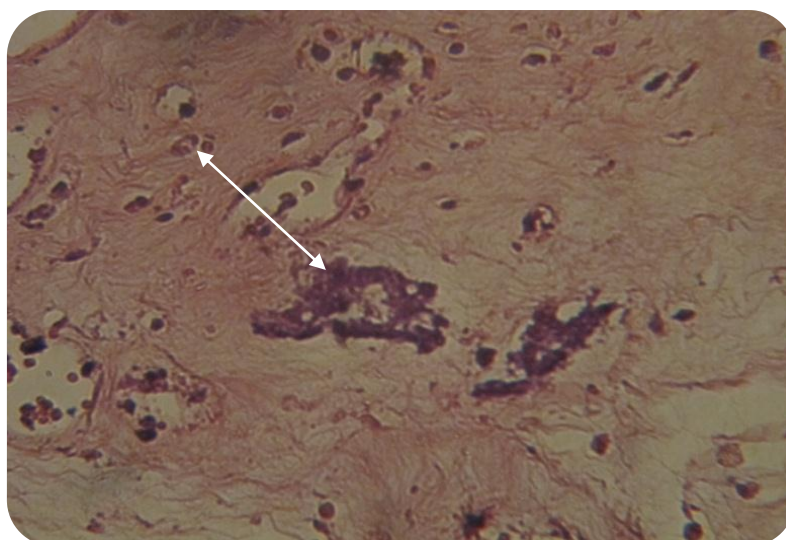


Fig:7. Histological section in the placenta of the aborted ewe showed calcium deposition in the placental plate with congestion of blood vessels(H&E stain, 400X)

Discussion:

According to the morphology of bacterial colonies ,gram stain and biochemical tests ,we suggested these isolates were *B. melitensis* which coincidence with PCR results, we recorded that all collecting samples from aborted ewes that showed positive Rose Bengal test expressed positive *Brucella* isolates and these bacterial isolates was variable according to area of sample collecting (Table:5).high bacterial isolates were recorded in the AL.manathera (44%) followed by Al shabaka(30%) and kuzweenah(26%) these results may be due to variation in the vaccination protocol or due to environmental condition that favor *Brucella* infection, also these results may be indicated that *Brucella melitensis* is widely distributed in these regions of AL-Najaf city and may be considered main causes of abortion in the ewes and these animals may be considered a main source of humans and other animals infection .This evidence was agreed with observations of(15).

Biotyping

25 out of 50 *Brucella melitensis* isolates, were *B. melitensis* biotype 1 , (50%), and 13 were *B. melitensis* biotype 2 (26%),12 were identified as *B. melitensis* biotype 3 (24%) after testing positive with mono specific anti A and anti M sera table(5), *Brucella* species and biotypes vary from country to country, even within the same country . The current study revealed that *B. melitensis* biotype 1 was the main biotype in the AL-Najaf city followed by biotype 2 and biotype 3 which was the less one. This result was in consistent with (17,18) who recorded that in various parts of the Iraq,Biovar types 1 ,2, and 3 were isolated from goat and sheep.

From the results of PCR amplification of the DNA of *B.melitensis* ,our explanation for the presence of multiple bands in Lines 2,3,4and 5 may be due to

polymorphism in *B. melitensis* gene, or these multiple bands belong to *B. melitensis* Rev.1 vaccine strain.

The results obtained by (19) ,confirmed these data, showing that DNA fragments obtained from *B. melitensis* strain biotype 1 should produce three fragments, an intact 282-bp fragment from the amplified *omp2a* gene that lacks the Pst1 site and two smaller fragments of 238 and 200 bp. In contrast, *B. abortus* vaccines strains (Rb.51, S.19) should produce only the two fragments from *omp2a* gene, a 550-bp fragment and a 200-bp fragment, respectively.

The strategy for the development of this PCR test is based on the observation that most organisms (prokaryotic and eukaryotic) contain strings of tandem repeated sequences classified as microsatellites and minisatellites distributed throughout their genomes that may affect protein expression. Tandem Repeated (TR) sequences are interesting class of markers, since multiple alleles can be present at a single locus, and size differences are easily achieved by electrophoresis. Tandem repeated sequences located within a repeated sequence and present in multiple loci were recently described and used for *Brucella* strain typing.

Confirmation by PCR ;

We demonstrated that all culturing bacterial isolates showed positive PCR assay fig (1). These results may indicated that the PCR assay was rapid and highly sensitive test for diagnosis of *B.melitensis* in aborted placental samples, these evidences were agreed with(21) who explained that the PCR is more sensitive and specific for detecting *Brucella* DNA as well as also useful for quantifying of the microbial load in acute and chronic infection .

In the present study, PCR assay was used for diagnosis of *B.melitensis* from pure culture, this result was in consistent with

(22) who found that the *Brucella* DNA can be detected by PCR from pure culture .

The histological result showed necrosis and desquamation of the trophoblasts ,this result may be due to the *B.melitensis* invaded these cells and lead to their destruction .These cells may provided good condition for *Brucella* growth ,this evidence was supported idea that mentioned by previous reports, (23) explained that during the early stages of infection, *B. melitensis* is found mostly in lymph nodes then the infection may progress to bacteremia and colonization of the uterus, where the organism replicates preferentially within trophoblasts in the rough endoplasmic reticulum also (24) showed that trophoblasts favor bacterial growth by producing erythritol and progesterone which stimulate growth of the *B.melitensis* .*melitensis* grows primarily in the extracotyledonary trophoblasts and then spreads to the cotyledonary (placental) trophoblasts .

The trophoblasts play essential role in protection of the fetal implantation and growth , there for ,we suggest that the destruction of these cells by *Brucella* may lead to abortion ,this investigation was agreed with (25) who explained that as a result of placentitis, fetal death, and abortion would be occurred during the last third of the gestation of bovine infection with *B.melitensis*.

We recorded suppurative placentitis in the present study ,this result may be indicated that the *B.melitensis* invaded phagocytic and nonphagocytic cells in the placenta and lead to stimulation of these cells to produce pro-inflammatory cytokines that attracting neutrophils to the site of inflammation, this idea was agreed with observation of (26) who found a significant upregulation of CXC chemokines, namely, CXCL6 (GCP-2) and CXCL8 (interleukin 8), by trophoblasts at 12 hrs after its inoculation with *B.abortus* and they suggested that the trophoblast

response is play a role in the pathogenesis of *Brucella* induced placentitis.

However, the infiltration of neutrophils in the inflamed area also lead to necrosis as a result of extracellular secretion their enzymes as well as occluding of the lumen of the blood vessels, these evidence may supported our result that revealed congestion of the blood vessels with neutrophils in their lumen, However, the pathological lesions in the aborted placenta in the present study were correlated with high level of placental TNF-alpha that observed by our immunohistochemistry results, these observations were in consistent with (27) who explained that the *B.abortus* associated molecular patterns such as lipopolysaccharide is recognized by Toll-like receptor4(TLR 4) and through MyD88 activated theNF-kB pathway, resulting pro-inflammatory cytokine induction also (28) mentioned that *Brucella* lipoproteins which are TLR2 ligands ,stimulated pro-inflammatory cytokine production.

The presence of dystrophic calcification in necrotic placentitis was agreed with results of (3)who showed calcium deposition in placentitis inducing by brucellosis ,

We detected severe pathological changes in the examined organs of the foetus characterized by fibrin networks deposition, neutrophils infiltration and these lesions were associated with *B.melitensis* isolation, these results may indicated that the microorganisms transmitted from placenta to fetus and induce damage fetal tissues, this idea was consistent with acute necrotizing suppurative placentitis associated with *B.melitensis* isolation from the placenta in the present study ,these results were agreed with (29) who found that infection trophoblasts with *Brucella* results in an acute inflammatory response.

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