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Brucella infection in cows and the associated pathological changes

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Abstract
The present study was carried out on a total number of 48 cows (14 aborted female at the late stage of pregnancy, 11 showed still birth, 7 retained placenta and 16 apparently normal female cows) the obtained sera were investigated for detection of *Brucella*-antibodies using serological tests. The results of serological analysis revealed that the positive reactors were 34(70.83%), 33(68.75), 32 (66.67%), 31(64.58) and 33(68.75%)using Buffer Acidifiedplate Antigen test (BAPA), Rose Bengal plate test (RBPT), Indirect ELISA (iELISA), Rivanol test (RivT) and Complement fixation test (CFT) respectively. Spleen, lymph nodes and uterus were collected from cows with positive serological reaction were subjected to bacteriological, molecular, histopathological and immunopathological examination.
*Brucella melitensis*biovar.3 was isolated from 26 out of 33serologically positive cows .Detection of Brucella DNA from serologically positive by PCR test from serum samples of corresponding cows recorded in 31(93.93%) cases and in tissue samples was 33(100%). The Genus-specific PCR assays detected a higher number of *Brucella* DNA from serologically positive cows compared with classical bacteriological culture methods. Regarding to histopathological studies, Lymph node revealed depletion of lymphocytes and proliferation of reticuloendothelial cells in lymphoid follicles, spleen showed dilation, thickening of splenic blood vessels with reticuloendothelial cells infiltration. Liver showed coagulative necrosis of hepatocytes, dilation and congestion of blood vessels with mononuclear cell infiltration in portal area. While uterus revealed granulomatous formation, some uterine gland showing necrosis and calcification. Immunopathological examination showed intense brown granules inside inflammatory cells in lymph node, spleen and uterus. The electron micrograph of supramammary lymph node of infected cow showing aggregates of dark bodies of intact coco bacilli within the cytoplasm of macrophage in the medullar sinuses. In this study, tissue samples should be used preferentially over serum samples for the molecular diagnosis of brucellosis The obtained results emphasize the importance of using more than one type of diagnostic technique for the detection of brucellosis in animals, especially with epidemiological purposes.

Keywords: *Brucella, serology, molecular, pathology, immunohistochemical.*

Introduction

Brucella are intracellular bacteria that cause brucellosis, a chronic disease of domestic and wild animals and humans. The ability of these bacteria to invade, survive for long periods of time and multiply within host cells is critical for disease causation **(Christopher *et al.*, 2010)**. The facultative intracellular parasitism characteristic of *Brucella spp*. evolved through evolutionary selection to avoid the host immune system. Target cells and tissues include trophoblasts, fetal lung, macrophages, and male and female reproductive organs **(Saxena, *et al.*, 2018).** Subsequently, fetal viscera and placenta become heavily infected, and placentitis and abortions occur, with devastating economic effects on livestock production. Although brucellosis is a widespread livestock infection in the Middle East and North Africa, it has not been studied in detail, only few stud-

ies showed rough figures about the epidemiology of the infection in these regions **(Wernery 2014).** The bacteria invade reproductive tissues, lymph nodes, and spleen, and therefore cause inflammation, edema, and necrosis. In pregnant animals it causes placental lesions that increases the risks of abortion **(Narnaware *et al.*, 2017).**

Brucellosis gains public health importance when the bacteria are transmitted to human via unpasteurized milk, meat, or animal byproducts, from infected animals **(Garcel *et al.*, 2016).** Proper diagnosis is one of the key obstacles for the complete eradication of brucellosis.

Diagnosis of brucellosis in cows is currently based on serological and microbiological tests. It is well known that serological methods are not always sensitive or specific **(Perry and Bundle, 1990).** Moreover, they have repeatedly been reported to cross-react with antigens other than those from *Brucella*spp. **(D´ıaz-Aparicio *et al.*, 1994).** Microbiological isolation and identification of the causative agents are the most reliable methods of diagnosing brucellosis. However, these procedures are not always successful as they are cumbersome and represent a great risk of infection for laboratory technicians **(L´opez-Merino, 1991).** Because of the high-level pathogenicity of the organisms, *Brucella* cultures must be handled with great caution. Nucleic acid-based detection methods, such as PCR, are very promising tools for diagnostics **(Fekete *et al.*, 1990)**. PCR provides a promising option for the diagnosis of brucellosis. It is a potentially useful method, either used alone or in combination with labeled probes, for the detection of *Brucella* spp. from isolated bacteria **(wang *et al.*, 2014)** or highly contaminated aborted tissues **(Fekete *et al.*, 1992).**

Here, we report a reliable, highly sensitive and specific single-step PCR test for the detection of *Brucellae* in tissue and blood obtained from infected cows. The report also describes a simplified method for extracting *Brucella* DNA from tissue and serum. This paper describes the evaluation of a single-step PCR for the detection *Brucella* DNA in tissues and serum of naturally infected cows. Its performance is compared with that of conventional serological tests (Buffer Acidified plate Antigen test (BAPA), Rose Bengal plate Test (RBPT), Indirect ELISA (iELISA), Rivanol test (RivT) and Complement fixation test (CFT) that performed on the same cattle serum samples analyzed by PCR. Histopathological examination was intended to confirm the result as well as detection of brucella antigen in lymph nodes, spleens and uterus of infected animals using the immunoperoxidase technique.

Materials and Methods

Animals and history

The present study was carried out on a total number of 48 cows obtained from farms located in El-Mansoura Governorate of known history of brucellosis (14 aborted females throughout the late stage of pregnancy, 11 showed still birth, 7 showed retained placenta16 apparently normal female cows. None of these animals were previously immunized against brucellosis.

Samples collection

Blood samples: Blood samples were collected from all examined cows then serum samples were separated and preserved at -20°C until used for serological assessments and extraction of DNA for PCR examination. Strict aseptic precautions were taken during collection of samples and disposable gloves were used for the collection of samples.

Tissue samples were collected from the lymph nodes ,spleen, livers, placenta, uteruses and mammary gland, of examined cows under sterile hygienic conditions and were immediately transported to the laboratory in a cooler with ice packs for bacteriological , PCR and Electron microscope examination. Parts from these tissue samples, were fixed in formalin (10%) for histopathological and Immunohistochemistry examination.

Serological examination

All sera were screened for antibodies against *Brucella* by Buffer acidified plate test (BAPA), Rose Bengal plate test (RBPT), indirect ELISA (as screening tests) and Rivanol test (RivT), Complement fixation test (CFT) (as confirmatory test) described by **Alton *et al*., (1988).**

Bacteriological examination
Specimens of retropharyngeal, supramammary lymph nodes, spleen and uterus were obtained from sero positive cows. Specimens were cultured on 7% blood agar (Oxoid, CM 271) and Brucella Medium (Oxoid, CM 169) supplemented with Brucella Selective Supplement (Oxoid, SR 209E). Cultures were incubated at 37°C for 5 to 7 days aerobically and micro aerobically under attention of 10% CO_2according to the method of **(Ribierio and Herr 1990).**

Pathological examination:
1-Macroscopic gross lesion
Complete post mortem examination was done on aborted dams to detect gross pathological lesion.

2-Histopathological examination
Small pieces from internal organs of aborted dams includlymph node ,spleen, liver, placenta, uterus and mammary gland, were fixed in 10% neutral buffer formalin solution for72 hrs then dehydrated in gradual ethanol (70-100%), cleared in xylene, and embedded in paraffin. Five-micron thick paraffin sections were prepared and then routinely stained with hematoxylin and eosin (HE) dyes and then examined microscopically. according to (**Bancroft *et al.*, 2013).** Special stainis used for detection of calcium as Von Kossa stain for calcification according to **(Sheehan and Hrapchak, 1980).**

3-Immunohistochemistry (IHC), was carried out according to the manufacturer's instructions using DAKO, an Agilent Technologies Company, USA, for detedtion of *brucella abortus* antigen in the infected tissue:
Paraffin-embedded tissue sections(4-5mm) are floatingin a 55 °C water bath and gently adhered to positively charged slides. Slides are then dried at 37 °C overnight. Deparaffinization and rehydration of tissue sections into the oven for 15 min at 60 °C, then air-dryed for 30 Sec. Slides placeed into xylene and incubated for 10 min to remove the paraffin, then 100% ethanol for 3 min towice, slides dipped in methanol H_2O_2 for 30 minutes, then 70% ethanol, and then slides washed with TBS towice at room temperature. Antigen retrieval with autoclaving. The deparaffinized, rehydrated slides placed in a glass staining dish and fill with TBS solution and completely covered with a glass lid and autoclaving at 121 for 15 min. Slides were transferred to room temperature and Tris-Buffered saline. Blocking of non-specific reaction: 5% skim milk in PBS was added to the slides and incubated at 37ºC for 30 minutes, then rinsed with PBS. Tissue slides with *B. abortus* antigen were soaked with polyclonal mouse anti-B.abortus and diluted 1:200 in PBS, incubated at 4ºC overnight, washed with PBS for 5 minutes 3 times. Yellow biotinylated link antimouse antibody (dako system universal) was added to the slides for 10 (±1) minutes washed with PBS for 5 minutes 3 times and dipped in distilled water twice then enough red drops of the Streptavidin reagent to cover specimen. Apply enough of the DAB Substrate-Chromogen solution to cover specimen. Incubate for 5–10 minutes. Rinse gently with distilled water .Immerse slides in a bath of Mayers hematoxylin. Incubated for two to five minutes, rinse gently in a distilled water bath for two to five minutes. Specimens mounted and coverslipped with an aqueous-based mounting Glycergel.
4-Ultra Structural Examination (Transmission electron microscope. TEM): Small tissue specimen was taken from supramammary lymph node and fixed in 5% cold cocodylate at 4^0C in ph 7.2 then kept at 4^0C until processing for electromicroscopic examination **(Bancroft and stenes,1982).**
Polymerase chain reaction (PCR)
Molecular Examination of serum and the cultures:
A. **DNA extraction.** DNA extraction from samples (serum and tissue) was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^OC for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

B. Oligonucleotide Primers. Primers used were supplied from biobasic (Canada) and are listed in table (1).

Table (1). Primers sequences, target genes, amplicon sizes and cycling conditions for conventional PCR.

Target gene	Target agent	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
					Secondary denaturation	Annealing	Extension		
1S711	Brucella genus	**IR1** GGC-GTG-TCT-GCA-TTC-AAC-G **IR2** GGC-TTG-TCT-GCA-TTC-AAG-G	839	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 50 sec.	72˚C 10 min.	**Bricker and Halling, (1994)**

C. PCR amplification.

Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

D. Analysis of the PCR Products.

The products of PCR were separated by electrophoresis on 1 % agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. A generuler 100 bp DNA Ladder (Fermentas, Thermo, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software

Table (2). Detection of *Brucella* specific antibodies response in the sera of examined cows by Serological tests

Examined animals	Serological tests									
	BAPA		RBT		iELISA		RivT		CFT	
	N.	%	N.	%	N.	%	N.	%	N.	%
Abortion (N= 14)	12	85.71	12	85.71	11	78.57	10	71.42	11	78.57
Stillbirth (N= 11)	11	100	11	100	11	100	11	100	11	100
Retained placenta (7)	6	85.71	6	85.71	5	71.43	5	71.43	6	85.71
Apparently normal female (N= 16)	6	37.50	5	31.25	5	31.25	5	31.25	5	31.25
Total (N= 48)	34	70.83	33	68.75	32	66.67	31	64.58	33	68.75

Table (3). Comparative results of culture and PCR in serologically positive and negative slaughtered cows

Serological status of examined animals	Bacteriological culture		Serum PCR		Tissue PCR	
	Positive	Negative	Positive	Negative	Positive	Negative
Serological Positive*(33)	26 (78.79%)	7 (21.21%)	31 (93.93%)	2 ()	33 (100%)	0 (0%)
Serological negative(15)	0 (0%)	15 (100%)	0 (0%)	15 (100%)	2 (13.33%)	13 (86.67%)
Total (N= 48)	26 (54.17%)	22 (45.83%)	31 (64.58%)	17 (4.17)	35 (72.92%)	13 (27.08)

*= **CFT** positive

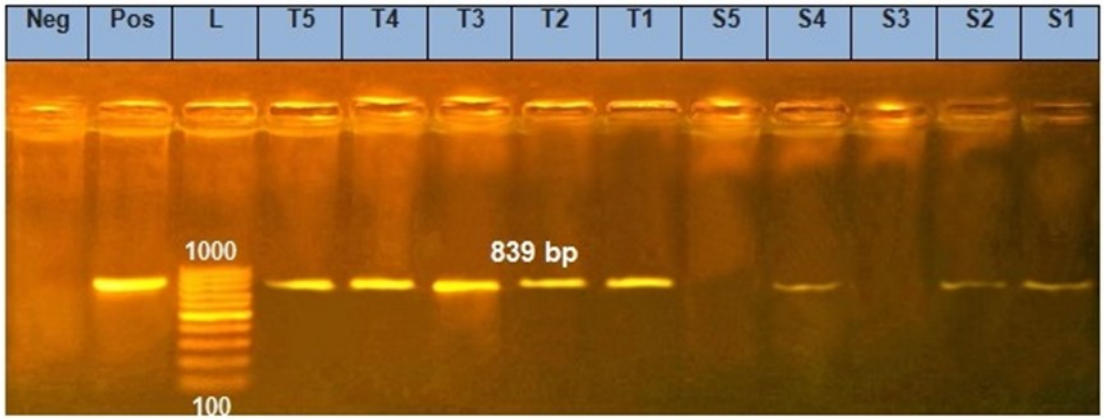

Fig. (1): Lane (L) molecular weight marker, lane (T1-T5) ***Brucella Genus***-DNA amplified from tissue samples (839-bp), lane S1.S2,S4 ***Brucella***-DNA amplified from serum samples, lane S3.S5 negative serum samples ,positive and negative control were included.

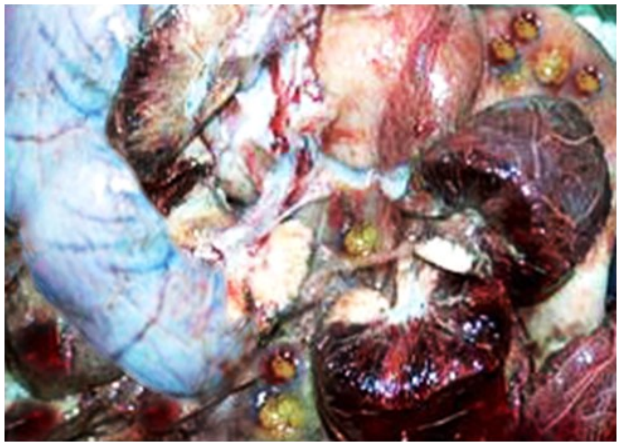

Photo. (1): Uterus of *Brucella* -infected cow after abortion shows Several necrotic and haemorrhagicplacentomes, severe and diffuse fibrinous exudate on the caruncular surface (acute placentitis).

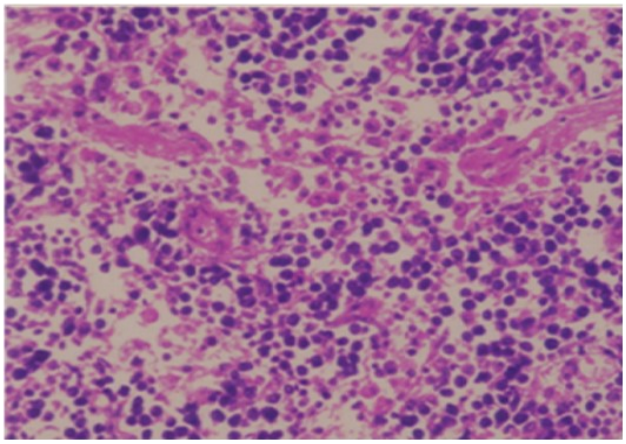

Photo. (2): .Lymph node of *Brucella* - infected cow showing depletion of lymphoid elements and activation of macrophages (H & E. X 400).

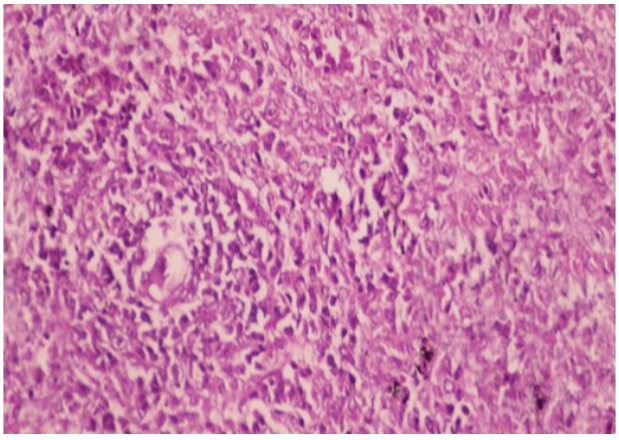

Photo. (3): Lymph node of *Brucella* - infected cow showing marked depletion of lymphocytes and proliferation of reticuloendothelial cells in lymphoid follicles. (H&E X 400)

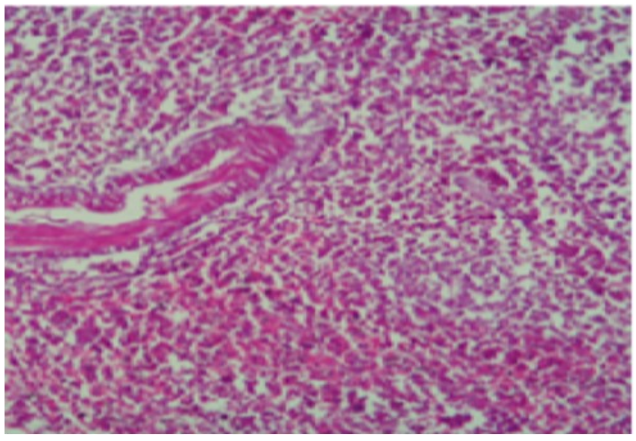

Photo. (4): Spleen of *Brucella* - infected cow showing dilation and thickening of splenic blood vessels with reticuloendothelial cells infiltration (H&E X100)

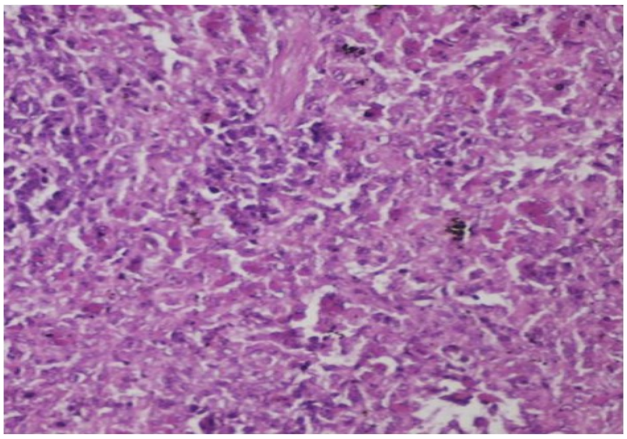

Photo. (5): Spleen of*Brucella* - infected cow showing increased cellularity in the cords of the red pulp, and proliferation of reticuloendothelial cells (H&E X 400).

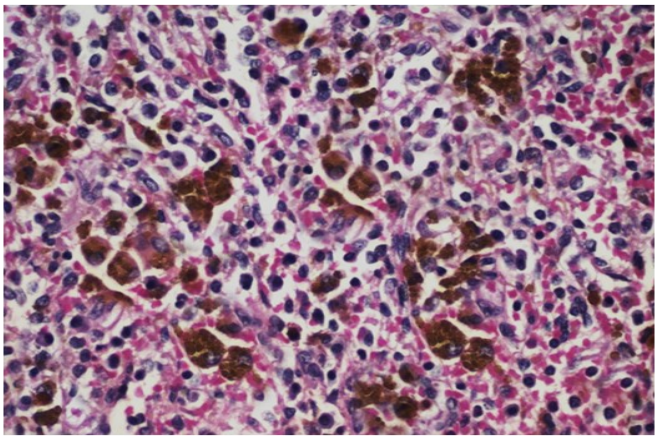

Photo. (6): Spleen of *Brucella*- infected cow showing haemosidrosis, (H&E, X400).

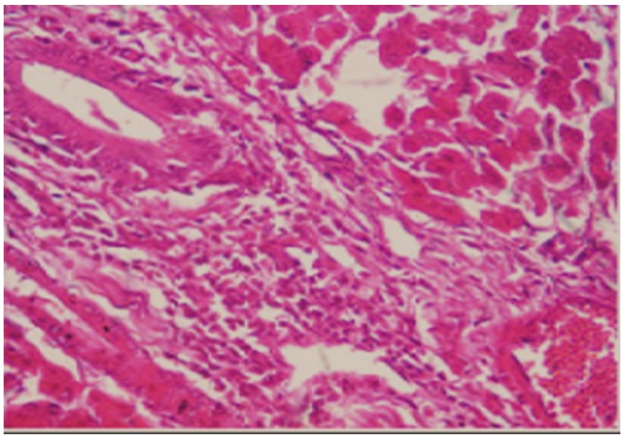

Photo. (7): Liver of *Brucella* - infected cow showing coagulative necrosis of hepatocytes, dilation of blood vessels with mononuclear cell infiltration in portal area (H&E X400).

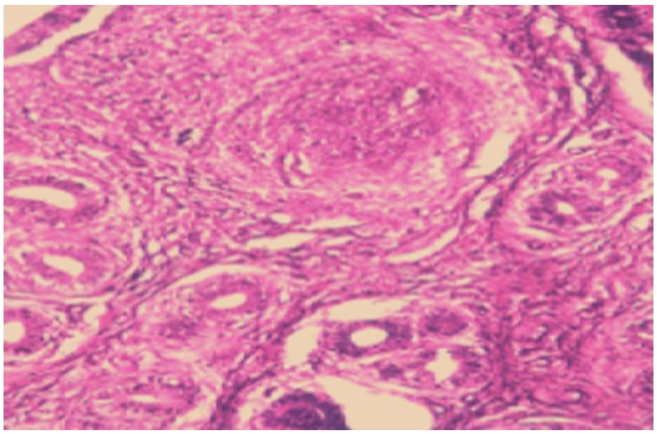

Photo. (8): uterus of *Brucella* - infected cow revealing the uterine mucosa showing necrosis of some uterine gland (H&E X200).

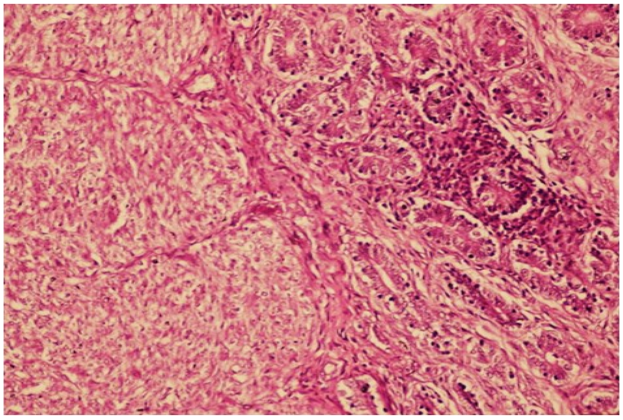

Photo. (9): Uteru sof *Brucella* infected cows showing edematous myometrium with vacuolated smooth muscle fibers. The interstitial tissue is infiltrated with mononuclear cells either in focal or in diffuse manner (H&E X 400)

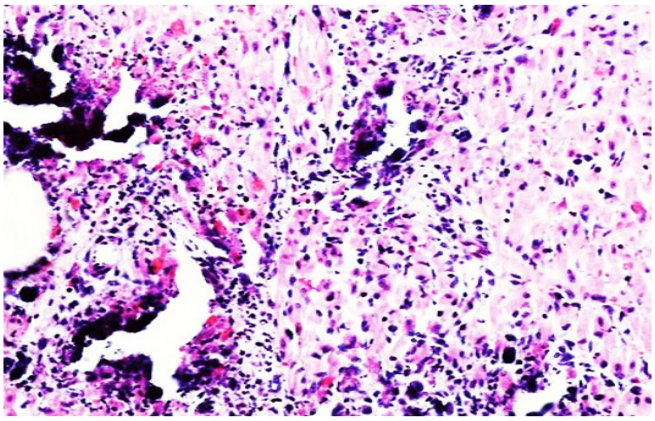

Fig. (10): Uterus of *Brucella* infected cows, showing brownish to black calcium deposition in the granulomatous structure. (Von Kossa stain, X400).

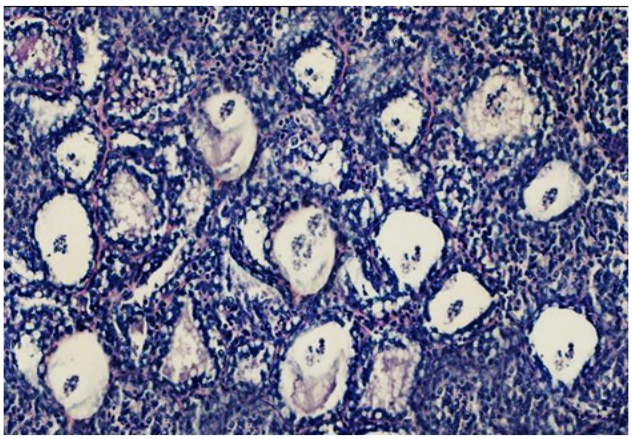

Fig. (11): mammary gland of *Brucella* infected cow, showing focal interstitial infiltration of lymphocytes, macrophages, and neutrophils and in acinar lumen. H &E,X100

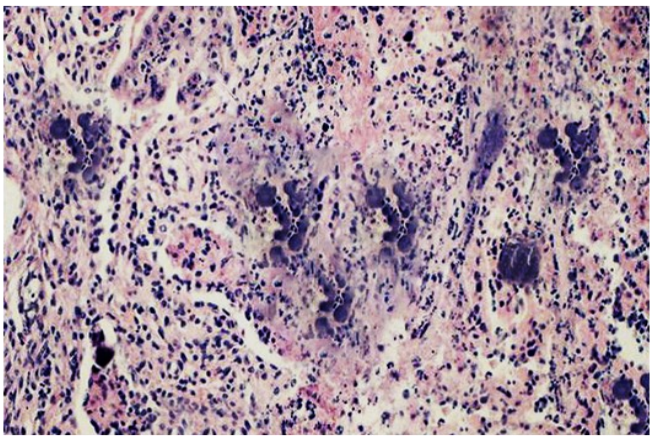

Fig. (12): Cow placentome of *Brucella* infected cow with caruncular crypts filled with necrotic debris, intense inflammatory infiltrate, and several bacterial colonies acutenecrotising placentitis.H&EX400

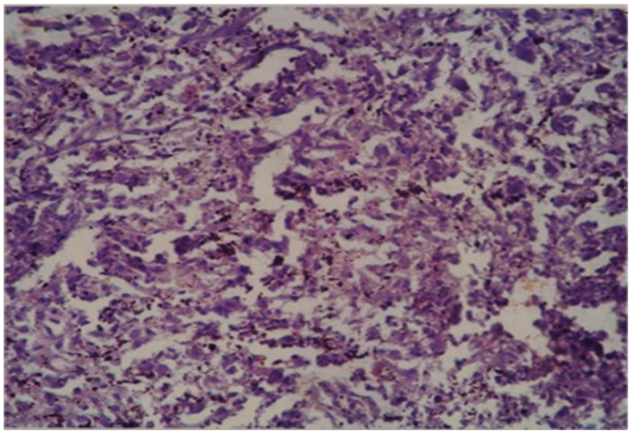

Fig. (13): Lymph node of *Brucella* infected cow revealed intense brown granules within interstitial tissue and lymphoid follicle. Immunoperoxidase.counter stain Mayer's hematoxylin . X100..

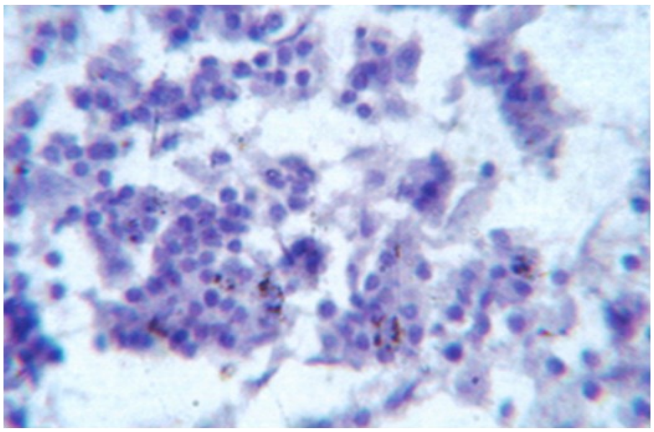

Fig. (14): Lymph node of *Brucella* infected cow revealed intense brown granules in cytoplasm of lymphocyte and macrophages. Immunoperoxidase. counter stain Mayer's hematoxylin . X400

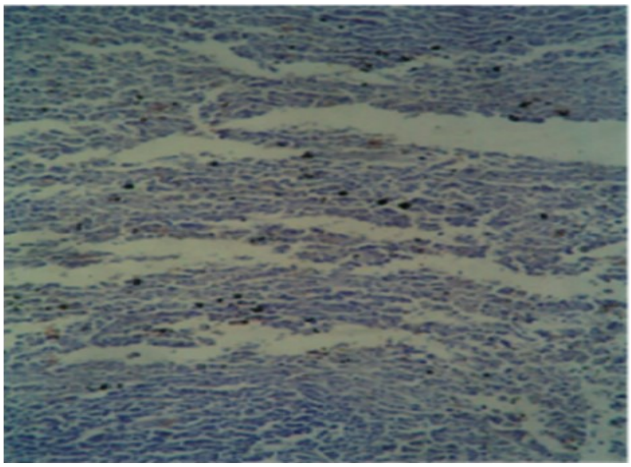

Fig. (15): Spleen of *Brucella* infected cow revealed intense brown granules within macrophage in lymphoid follicles. (Immunoperoxidase. counter stain Mayer's hematoxylin) . X100.

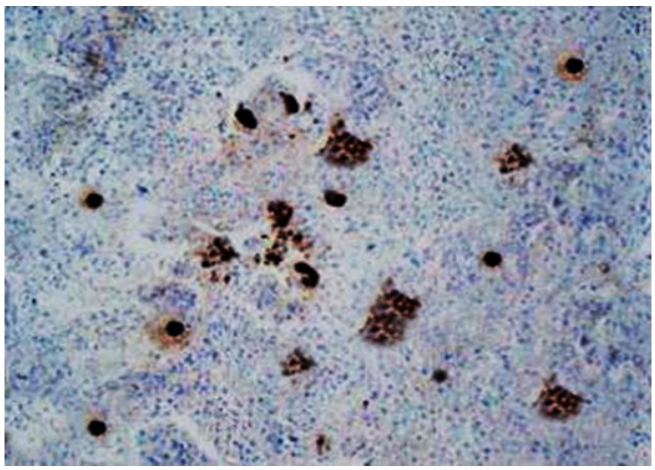

Fig. (16): placenta of *Brucella* infected cow shows multifocal brown distribution of several colonies of B. abortus (Immunoperoxidase. counter stain Mayer's hematoxylin). X100

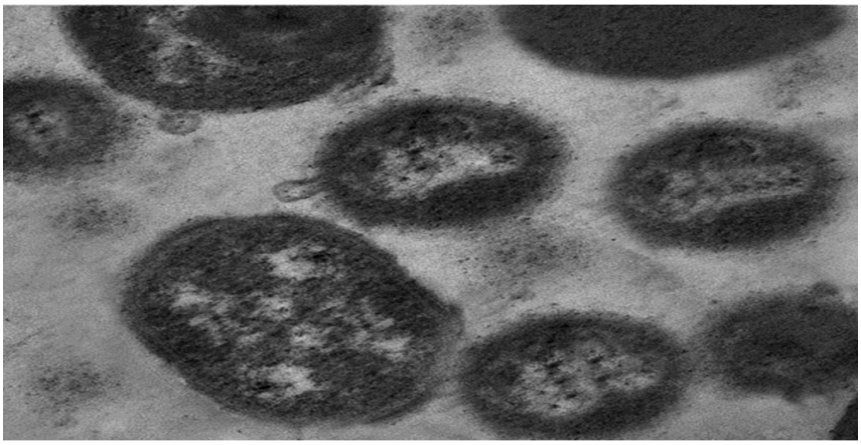

Fig. (17): Ultra Structural Examination (Electron micrograph) of supramammary lymph node of Brucella infected cow showing aggregates of dark bodies of intact coco bacilli within the cytoplasm of macrophage in the medullarysinuses. X100,000

Results and Discussion

Bovine brucellosis is considered to be a great problem as a cause of abortion in many countries in the world**.** The resistance of animals to Brucella infection is influenced by sex, age and reproductive status **(Ducrotoy *et al.*, 2018).**

Pregnant animals are more susceptible to infection than young one**.** Brucella organisms first localized in regional lymph node, they proliferate within reticuloendothelial cells with subsequent entry into lymphatics and localized in different tissues like spleen and reproductive organs **(Foster *et al.*, 2017).** Abortion is a frequent complication of brucellosis in animals, where placental localization is believed to be associated with erythritol, a growth stimulant for *B. abortus.* Results of clinical sings of aborted cows were similar to the results of many researches who explained that abortion in cattle due to brucellosis occurred at late stage of pregnancy and may result in the birth of dead calves and retained placentas **(Gabli *et al.*, 2015).** It has been postulated that generalized suppression of adaptive immune response mainly occurred during pregnancy. This immune suppressed state prevents maternal rejection of the fetus but has unfortunate consequence of increasing maternal susceptibility to certain infectious agents **(Krishnan *et al.*, 1996).**

The presumptive diagnosis provided by the serological tests, is usually accepted as indication of brucellosis. Buffer Acidified Plate Test (BAPT), Rose Bengal Plate Test (RBT), Rivanol test (RivT), indirect ELISA (i ELISA) and complement fixation test (CFT), are utilized in this study for the detection of antibodies specific to *Brucella* spp. **(Pandeya *et al,* 2013).**

As regards to the results presented in table (1) 34 (70.83%), 33(68.75), 32(66.67%), 31(64.58 and 33(68.75%) cattle were positive by BAPAT, RBPT, RivT, iELISA and CFT respectively. The results show that BAPAT is more sensitive than RBPT. So, it is concluded from table (1) that conventional agglutination tests have good sensitivity but lack specificity and the occurrence of false positive serological results make a specific test necessary which agrees with **Bronsvoort *et al*., (2009)** who stated that although some diagnostic or screening tests are referred to as the "gold standard" but it need the use of a more specific test to confirm any positive animals. The BAPAT test detected a higher number of positive as compared with other serological tests and this attributed to the final pH of serum antigen mixture of 4.02 ± 0.04 **(Alton *et al*., 1988),** this pH enable some of the IgM besides IgG1, IgG2 and IgA to share in the reactions (**Wright and Nielsen, 1990**). Also superiority of BAPA rather than RBPT may attributed to the amount of serum used in BAPA (0.08 ml) is greater than the amounts of serum used in the RBPT (0.03 ml) and due to the relatively acidic pH of the RBPT (3.65) permits lesser amounts of IgM to share in the reaction, since this class of immunoglobulin is known to be acid labile **(Allan *et al.*, 1976).** Furtherly **OIE, (2009**) reported that buffered Brucella antigen tests are

suitable for screening herds and individual animals
Application of Rivanol test in this study revealed that it detected positive reactors lower than those detected by BAPA or RBT. Our result are in agreement to those reported by **Hamdy (1992) and Hosein *et al*., (2002)**.

The lower incidence obtained by Riv.T than that BAPA and RBT may be due to the use of Rivanol solution (2 – ethoxu- 6, 9 diaminoacridine lactate) to serum that selectively precipitates all serum proteins except IgG. The concentration of Rivanol solution was standardized to precipitate the IgM class of antibodies which include the non specific agglutinating materials from bovine serum as reported by **Pietz and Coward (1980)**.

In the present study, iELISA provided positive reactors less than RBPT. Similar findings given by **(Hermoon *et al.,* 2001)** who reported that ELISA has been shown to be suitable test for large scale screening for bovine brucellosis. Besides, latent infection could be detected earlier by ELISA than other serological tests as it detect all classes of antibodies**.**

Conventional serologic methods such as agglutination tests and primary binding assays such as i-ELISA, principally measure antibody to S-LPS either as presented on the intact bacterium or immobilized on a plastic matrix. The antibody response of animals to S-LPS from smooth vaccines or field strains decrease by time but antibody titers persist longer in naturally infected *animals* **(Nielsen 2010**). So, these conventional serological tests have limited ability to discriminate vaccinated from naturally infected animals **(Crasta *et al.,* 2008).** Also **Marin *et al.,* (1999)** suggested that the similarity in the S-LPS response hampered diagnostic efforts to differentiate vaccinated from infected animals

CFT is considered as gold standard serological test used for detection of brucellosis as it detect only IgG specific for *Brucella* infection so it overcome cross reaction with other similar gram negative bacteria and so no false results detected. Additionally, World Organization for Animal Health (OIE) suggested that CFT is a test approved all over the world for testing animals for international trade **(OIE 2009).** This test is considered as a high-quality test when correctly used, however it has lots of practical drawbacks such as time consuming and difficult to standardize **(Abernethy *et al.,* 2012).**

Confirmatory diagnosis must be provided by the isolation of etiological agents. Therefore, the isolation of *Br. melitensis*is important to study the epidemiology of brucellosis.

Brucellae melitensis biovar 3 was isolated from 26 (54.17%) cattle out of 48 serologically positive by CFT. These findings are come in accordance with **Montasser, (1991)** who reported that tissue samples which gave negative results may be due to the longtime required for culturing field specimens can be and /or tissues are contaminated with a low number or nonviable *brucella* organism. Despite of *brucella* isolation and identification are direct and reliable methods for diagnosis, they are time consuming, cumbersome and represent human health hazard.

Previous studies in various parts of Egypt indicate that the *B. melitensis* biovar 3 is the most prevalent field strain (**Montasser, 1991)** The isolation of *B. melitensis* strains indicated very high prevalence of *B. melitensis* infection among these animals in this region and due to that, the disease may threat human and animal health which was coincide **(Esmaeil *et al*., 2008).**

In this study, Detection of Brucella DNA from serologically positive cows by PCR test from serum samples of corresponding cows recorded in 31(93.93%)cases and in tissue samples was 33(100%).Only 2 serologically negative cows were positive by PCR from tissue samples.

The advantages of PCR technique is fast, safe and unaffected by contamination by other microbes that might be present in the tissue samples used for isolation, that explain the superiority of the PCR assay as a diagnostic method of brucellosis in serum and tissues of infected cows and failure of serological test to recognized and detect

brucella recently in aborted cows **(Da Silva Mol *et al.*, 2012).** This study discovered the using of PCR that can be beneficial for detection of *Brucella* DNA in seronegative animals. Serological diagnosis from freshly aborted animals may fail because low antibody titers against *Brucella* infection. or absences of antibody in some animals sera **(Habtamu *et al.*, 2013**).

In this study, PCR done on tissue samples for examined cows gave a higher proportion of positive results than serum PCR. This results coincided with **Gemechu *et al.*, (2011).**who suggest that PCR analysis of the supramammary and mammary lymph tissue is a better way to detect *Brucella* DNA than analysis of the serum samples. This is important for the detection of *Brucella* by PCR using lymphoidtissue samples from postmortem cows. There may be number of reasons why tissue PCR for the detection of Brucella DNA is superior to serum PCR. The stage of infection may influence the number and location of *Brucella* organisms in white blood cells and lymphoid tissue glands .Indeed, we did not know the stage of infection of cows in this study. However, the cows were diagnosed as positive for *Brucella* antibodies by CFT and therefore, they evidently had an increased humoral immune response for some time. Whereas. Animals excrete Brucella organisms in their blood at the early stages of the infection, materials used in this study might have been collected during later stages of the infection. It should be borne in mind that the serologically positive samples with PCR positive results contained infectious bacteria and this has clinical environmental significance. An ideal diagnostic method should be specific, sensitive, fast, inexpensive and easy to perform. At present the PCR assay described fulfils all these requirements except that it is fairly expensive, but reagent cost may decrease as more PCR diagnostic assays are developed. PCR has a significant safety advantage over culture, and could provide results within few hours. In conclusion, we emphasize the importance of using more than one type of diagnostic technique for the detection of brucellosis in animals, especially for epidemiological purposes **(Molavi *et al.*, 2017).**

Macroscopical alterations of infected cows revealed enlargement in spleen with petechial hemorrhage on the splenic capsule and whitish necrotic foci in other cases. Lymph nodes appeared moist consistency, enlarged and edematous. Uteri revealed the presence of moderate amount of mucous exudate and multiple white necrotic foci in the endometrium. Immediately after abortion, severe and diffuse haemorrhagicacute placentitis. Cow place tome with caruncular crypts filled with haemorrhagic necrotic debris and intense inflammatory infiltrate (photo.1). Enlargement of udder with some turbid exudate. This agree with **Adams (2002) and Poester *et al.* (2013)** who reported that The pathological changes observed in different organs may be attributed to continuous exposure of animal to microorganisms, intermittent disseminations of organism in blood from old lesion and the responsive effect of the immune reaction

Histopathologically, lymph nodes revealed marked lymphocytic depletion, proliferation of reticuloendothelial cells in lymphoid follicles and activation of macrophages (photo. 2 and photo. 3). These may attributed to the main immunological function of lymphoid tissue which represented by guard the body's vascular compartment by generating T cells and dependant IgM antibody responses to bacterial polysaccharides and by exerting an enomous phagocytic power before antibody formation and subsequent opsonization (**Schlafer and Miller, 2007) and (Carvalho *et al.*, 2010)**. The previous study revealed that brucella can induce the release of pro- inflammatory cytokines **(Adams, 2002).**

Spleen showing dilation and thickening of splenic blood vessels with reticuloendothelial cells infiltration (photo. 4) as well as increased cellularity in the cords of the red pulp,and proliferation of reticuloendothelial cells (photo.5). Investigation of changes in reticuloendothelial cells during course of infection from onset to resolution may provide insights on the ability of brucella to stimulate the host response and revealed a pattern for the organisms to the localized in these organs which most not ably in spleen and liver **(Merk *et al* 2004).** Patches of irregular shapes of haemosidrosis were ob-

served as brown precipitations of iron pigment in white pulp of spleen (photo.6).

The lesion in liver revealed coagulative necrosis of hepatocytes, dilation and congestion of blood vessels with mononuclear cell infiltration in portal area (photo.7). Infected macrophages underwent oncosis, which is prelethal pathway leading to cell death characterized by cell organella swelling, cell bulbing and increase cell permeability **(Fink and Cookson 2005).** On other hand **Ahmed *et al* (2012)** explained that the brucella is incorporated into phagosomes and remains in membrane bound compartment until the host cell dies. The ability of brucella to survive in intracellular environment is apparently due to the inhibition of phagosom–lysosome infusion that give the ability of pathogen to invade phagocytic and non-phagocytic host cell **(Arenan *et al* 2000).**

In the present study, the histopathological changes in uterus were characterized by edematous myometrium with vacuolated smooth muscle fibers. The interstitial tissue is infiltrated with mononuclear cells either in focal or in diffuse manner (photo. 8 and 9). These results were explained by **(Beruktayet and Mersha, 2016)** who said that brucella release pro inflammatory cytokines in a variety of cell types such as tumour necrosis factor alpha (TNF) that is required for the influx of phagocytes to the site of infection for macrophage activation **(Rust *et al.,* 2004) and (Holt *et al.,* 2011)** thus, potent cytokine stimulatory properties possessed by brucella sp. may explain the correlation between tissue invasion and localized inflammation. Moreover presence of high amounts of erythritol in uterine tissue attracts the microorganism leads to the inflammation in the uterus.
In avariety of cell types such as interlukines (IL-12) and tumor necrosis factor alpha (TNFα) (**Zahn *et al* 1996)**.Thus potent cytokines stimulatory properties processed by brucella may explain the correlation between tissue invasion and localized inflammation. Sections stained with Von Kosa stain revealed brownish black coloration in the center of granuloma (photo.10).

Mammary gland shows focal interstitial mastitis and infiltration of lymphocytes, macrophages, and neutrophils in anacinar lumen (photo.11), that inagreement with **Carvalho *et al.,* 2010)** who reported that**,** the mammary gland is another target organ that is important intransmitting the infection through contaminated milk. It was proved that B. abortus induces a multifocal interstitial mastitis with interstitial accumulation of macrophages and intra-acinar infiltration of neutrophils associated with moderate numbers of predominantly intracellular organisms **(Meador *et al.,*1989)**.

Placenta with caruncular crypts filled with intense inflammatory infiltrate necrotic debris, (acutenecrotising placentitis)(photo.12), these lesion explain the chronicity of brucella infection which represented by fibrous invasion and calcification in uterus and agree with results obtained by **Madbouly *et al.,* (2012).**

The specific granular immunohistochemical staining to the anti -*B. melitensis* serum was detected mainly within macrophages and interstitial tissue in lymphoid follicle found inlymph nodes (photo .13 and 14). Spleen of cow revealed intense brown granules within macrophage in lymphoid follicles (photo 15). Placenta shows brown immunolabelled several colonies of *B. abortus* with a multifocal distribution (photo. 16). Similarly, previous reports shown that organisms were located mainly in the cytoplasm of the macrophages in the inflammatory foci. The immunore activity pattern is located mainly in the cytoplasm of macrophages and cellular debris **(Pérez *et al.* 1998).**

The immunohistochemical technique in our study was sufficiently sensitive for detecting Brucella antigens in formalin fixed tissue. This agree with (***Vincent et al. 1997)*** who reported that the immunohistochemical test may be useful for performing retrospective studies because antigen detected by this method have been shown to be stable in paraffin section for 10 years. This immunohistochemical technique could be a complementary tool to serology and bacteriology for diagnosis of brucella infection. Moreover, **Staak *et al.,* (2000)** reported that immunoperoxidase technique may enhance diagnosis capabilities of brucellosis par-

ticularly in chronic infection and is an efficient mean for detecting *Brucella* organisms when are inherently slow or difficult to diagnose by isolation or culture from tissues obtained from field cases due to contamination. In addition, this technique is relatively rapid and enables detection of dead and/ or low numbers of bacteria **(Haines and West, 2005)** although, cross reaction of the polyclonal antibodies with other microorganisms such as *Yersinia enterocolotica* and *E. coli* cannot rule out. Also, immunohistochemical staining has been used to study and assist in the understanding of the pathogenesis of infectious agents as the quantity, tissue and cellular locations of agent can be visualized.

The ultra-structural finding showed presence of aggregates of dark bodies of intact dense cocci or cocobacilli within the macrophages (photo. 17) which means the ability of the organism to survive intracellularly **(Arenas *et al.*, 2000 and Pei *et al.*, 2006)**. The ability of brucella to survive in the intracellular environment is apparently due to inhibition ofphagosome-lysosome fusion **(Arenas *et al.*, 2000)**. in addition tonecrosis, brucella -infected macrophages under wentoncosis, which is a prelethal pathway leading to cell death characterized by cell organelle swelling, cellblebbing and increased membrane permeability **(Fink and Cookson, 2005)**. These findings were confirmed by previous reports indicating that infected cells were not killed via apoptosis **(Pei and Ficht, 2004)**. The organism can reach replication niches and survive and the host cells will be killed. Otherwise the bacteria will be cleared by the host cells **(Pei *et al.*, 2006).**

Finally, it could be concluded that various laboratory methods have been used or introduced for the detection of *Brucella*. Molecular methods such as PCR, is a sensitive, specific, rapid, relatively inexpensive method for detection of bacteria and could be accurate method for diagnosis of Brucellosis, thereby could control the infectious diseases in cows and minimize reproductive losses. Moreover, immunoperoxidesetechnique enhances the diagnostic capabilities of brucellosis particularly in chronic infection in cows.

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