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COMPARATIVE STUDIES FOR DETECTION OF BRUCELLA MICRO-ORGANISM IN ABORTED BOVINE FOETI USING CONVENTIONAL, IMMUNOHISTOCHEMICAL AND MOLECULAR METHODS

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ABSTRACT

The present study was carried out on a total number of 78 bovine (cows48- Buffaloes 30) The obtained sera were investigated for detection of *brucella*-antibody titer using serological tests Rose Bengal plate Test (RBPT), Tube agglutination test (TAT), indirect ELISA (iELISA) and Complement Fixation Test (CFT). Abomasal content were collected from 78 aborted fetuses for bacteriological and Polymerase Chain Reaction (PCR) assays. The results of serological analysis revealed that the positive reactors were 60 (76.92 %), 55 (70.51 %), 58 (74.36 %) and 57 (73.08 %) using RBPT, TAT, iELISA and CFT, respectively. RBPT and iELISA tests showed the highest seropositivity. Meanwhile, the lowest ones were obtained by TAT and CFT tests. *Brucellaem elitensis* biovar3 was isolated from 31 out of 48cows (abomasal content) aborted fetuses and21 out of 30buffaloes (abomasal content) aborted fetuses. PCR assay for detection of Brucella in aborted fetuses were 33 out of 48 in cow and 22 out of 30 in buffaloes. Pathological examination in organs of aborted fetuses infected by *Brucella melitensis* showed significantly pathological lesions in lungs, liver, spleen and placenta. Immunohistochemistry revealed the presence of positive immunostaining *brucella* antigen in formalin-fixed, paraffin imbedded tissue sections of lung, liver, spleen, and placenta by using avidin–biotin complex peroxidase technique. Electron microscopical finding of lung, liver and spleen showed that the cytoplasm of neutrophil and macrophages containing dark bodies of cocobacilli. TEM of placenta revealed that the Trophoblasts filled with degenerated organisms.

Key words: *Brucella*, *serology, molecular, pathology, immunohistochemical.*

INTRODUCTION

Brucellosis is a prevalent zoonotic disease affecting both humans and animals caused by bacteria of the genus *Brucella* (WHO/OIE/FAO/CDS 2006). It is found to be one of the most common public health problems all over the world (Kardjadj *et al.,* 2016). Brucellae are facultative gram negative intracellular bacteria of genus Brucella which are survivors in both extracellular and intracellular environments. The main domestic animals that are affected are cattle, sheep, goats and pigs, (Nicoletti and Tanya, 1993). It is known to be a worldwide problem and one of the most important among zoonoses in the Mediterranean region, India, and Central and South America (Ashford *et al.,* 2004). Outbreaks of bovine brucellosis are associated with abortion during the last trimester of gestation, and produces weak newborn calves, and infertility in cows and bulls (Megid *et al.,* 2010).

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Precise diagnosis of livestock and humans brucellosis is considered the keystone for its correct abolition and manages. In general, diagnosis of brucellosis is somewhat difficult as the disease may have an incubation period varying from 5 days to 5 months and can progress in various forms: acute, chronic or as ymptomatic (Nimri, 2003). To reduce economic losses from brucellosis, accurate, safe and sensitive diagnostic methods play avital role in the control and eradication program of brucellosis in animals and humans. The gold standard for the diagnosis of brucellosis is the isolation of the pathogen. However, isolation of the organism is time consuming and resource-intensive. Organism handling also requires specialized laboratory, bio-containment facilities and highly skilled personnel to handle clinical samples and live bacteria for eventual identification, speciation and biotyping (Kaltungo *et al.,* 2014). Consequently, serological assays are frequently used for diagnosis of animal brucellosis particularly in cattle, sheep, goats and camels but cross-

reactions with other pathogens including *Yersinia enterocolitica, Salmonella genus, Escherichia coli* O:157 and other gram negative bacteria till now represent a big problem (Nielsen *et al.,* 2004).

In order to overcome these difficulties, polymerase chain reaction (PCR) based assays have been developed for the rapid identification and confirmation of microbes including Brucella, which almost completely obviate the need for direct handling of the pathogen. PCR has been developed for the detection of *Brucella* in a wide variety of clinical samples such as aborted fetuses (Buyukcangaz *et al.,* 2011) and lymphoid tissue (Ilhan *et al.,* 2008) and has been introduced as an accurate and sensitive assay for detection of *Brucella* spp. Alternative methods for the detection of *Brucella* organism in tissues include Immunohistochemical examination of paraffin wax- embedded tissues which is not only both sensitive and specific but also clearly shows tissue morphology; it is, therefore, capable of demonstrating the distribution of organisms in the tissues, a valuable attribute for the study of pathogenesis of *B. abortus* infection (Meador *et al.,* 1986 and Perez *et al.,* 1998).

Despite the vigorous attempt for more than one century to come up with a definitive diagnostic technique for brucellosis, diagnosis still relies on the combination of several tests to avoid false negative results (Poiester *et al.,* 2010).

The aim of this study was planned to: (i) compare the diagnostic performance of serological standard RB and iELISA and CFT in aborted bovine, and molecular PCR studies on abomasal content of aborted fetus with known bacteriological status (ii) study the histopathological changes in the internal fetal organs and placenta of dams infected with brucella, in addition detection of the organism in infected tissues by immunohistochemical and electron microscopical techniques.

MATERIALS AND METHODS

Animals and history

The present study was carried out on a total number of 78 animals including 48 cows and 30 buffalos obtained from farms located in El-Gharbia Governorate of known history of brucellosis. These animals suffered from abortion in late stage of pregnancy. None of the animals were previously immunized against Brucella.

Samples collection

A. Blood samples:

Seventy eight blood samples (48 from cows and 30 from buffalos) were collected and then serum samples were separated and preserved at -20°C until used for serological assessments. Strictaseptic precautions were taken during collection of samples and different disposable gloves were used for the collection of each sample.

B. Aborted fetuses:

Aborted fetuses from serologically positive animals for brucellosis were used. Abomasal contents of aborted fetuses which were collected under sterile hygienic conditions and were immediately transported to the laboratory in a cooler with ice packs for bacteriological and molecular examination. Internal organs of the fetuses which included liver, spleen, lung and placenta were collected for Pathological, Immunohistochemistry and Ultra structural examination.

Serological examination

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

Bacteriological examination

Abomasal contents were cultured on 7% blood agar (Oxoid, CM 271) and *Brucella* Medium (Oxoid, CM 169) supplemented with *Brucella* Selective Supplement (Oxoid, SR209E). Cultures were incubated at 37°C for 5 to 7 days aerobically and microaerobically (Microaerobic kit, Merck, Anaerocult C) according to the method of (Ribierio and Herr 1990).

Pathological examination:

Complete post mortem examination was done on aborted fetuses to detect any gross pathological lesions. Small pieces from internal organs of the fetus of aborted bovine positive by using CFT (57) which included liver, spleen, lung 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 (Bancroft *et al.,* 1996).

Immunohistochemistry

Brucella melitensis antigens were demonstrated by using the avidin-biotin-peroxidase complex immunehistochemistry staining method. Formalin-fixed, paraffin embedded tissue sections of lung, liver, spleen and placenta on coated positive slides were used and deparaffinised in xylene for 10 min and rehydrated through a series of graded alcohol. Heat mediated antigen retrieval was used by

immersing the slides in citrate buffer solution and heated in microwave oven for 10 min at low(55-60°C) temperature. Blocking with 3%hydrogen peroxidase for 5 min at room temperature inactivated the indigenous peroxidase. The sections were washed with PBS and blocked with 5% bovine serum albumin (Gibco, USA) for 15 min at 37˚C, followed with overnight incubation at 4˚C with primary poly colonal anti brucellamelitensis antibody prepared in rabbit (1:100) (Difco. lab. USA). The sections were then incubated with secondary antibody (IgG goat anti rabbit) (Abcam, UK) for 1 h at 37˚C (1:500). The slides were rinsed and washed with PBS before diaminobenzidine DAB (Dako, USA) was used as chromogen for 15 sec. Mayer'shaematoxylin stain was used as counter stain and covered with glycerin gell and examined by light microscope. The degree of IHC staining was scored as strong positive brown staining (+++ve), moderate brown staining, (++ve) and mild brown staining (+ve) (Haines, and Clark, 1991).

Ultra structural Investigation:
Small pieces of 1mm from lung, liver, spleen and placenta from all examined aborted bovine positevly by using CFT (57) were collected washed, fixed with 3% glutraldehyde, and processed for transmission electron microscopy according to (Bancroft and Stevens 1982 and cheville *et al.,* 1996) and examined using JEOL –JEM.1400 Electron Microscope at Faculty of Agriculture Research Park (FARP).

Polymerase chain reaction (PCR)
DNA extraction:
From each animal, 10 ml of abomasal contents of aborted fetuses were collected by 21G sterile needle. DNA extraction and purification were performed according to the method of (Fekete *et al*., 1992).

Oligonucleotide primers: B. abortus, B. melitensis and IS711 primers sequence used as described (Bricker & Halling, 1994).

Table 1: Sequences of the oligonucleotide primers used in PCR as say.

Primer	Sequence (5´-3´)
B.abortus-specific primer	GAC-GAA-CGG-AAT-TTT-TCC-AAT-CCC
B.melitensis-specific primer.	AAA-TCG-CGT-CCT-TGC-TGG-TCT-GA
IS711-specific primer	TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT

Amplification of Brucella-DNA and detection of PCR products. PCR conditions were performed as described by (Bricker and Halling 1994).

DNA amplification in conventional PCR: DNA amplification was done in 25 ul reaction volume containing 5 ul of Taq master ready–to–use mixes for PCR (Jena Bioscience, Cat No. 102S), 10 PM of each oligonucleotide primers, 5 ul of DNA template and fill up to 25 ul with molecular grade water. The optimized cycling conditions consisted of 40 cycles of 1min. at 95ºC, 2 minute at 60ºC and 1min. at 72ºC; and final extension step at 72ºC for 5 min. (Bricker & Halling, 1994). The negative control contained sterile water instead of DNA template, while, the positive controls was DNA isolated from *B. melitensis* Rev1 were used.

Electrophoresis of PCR product: Amplification PCR products was analyzed by electrophoresis through 1.5 % agarose gel stained with etidium bromide solution (0.5 mg/ml) and visualized under an ultraviolet transilluminator and photographed. Visible bands of PCR products with the molecular sizes of 498 and 731 bp were considered indicative for identification as *B. abortus and B. melitensis,* respectively.

RESULTS

Serological, bacteriological and molecular assessments:
Serological examination for incidence of *Brucell* aspp. among aborted cows and buffaloes using RBPT, Tube agglutination test (TAT) and iELISA as screening tests and CFT as confirmatory test revealed that 60 (76.92%), 55 (70.51%), 58 (74.36%) and 57 (73.08%) samples were positive result respectively (Table 2). *Brucellae melitensis* biovar 3 was isolated from 52 (abomasal content) of aborted fetuses (31cows and 21 buffaloes). PCR assay for detection of Brucella DNA in aborted fetuses were positive in 55 examined animals (33 cows and 22 buffaloes) (Table 3).

Gross lesions: In this study the abortions occurred in the seventh and eighth months of gestation. Gross lesions were present in all naturally infected fetuses. Differences in the character or distribution of lesions in these fetuses were not noticed. Their subcutaneous tissues were oedematous and their thoracic and abdominal cavities contained an excess of thin red-tinted fluid. Most of the infected fetuses had

changes in lungs, which observed purpletotan, firm areas were noticed throughout affected lung lobes. In several fetuses, both caudal lobes were entirely involved. The lobes were gray, firm and enlarged with indentations from adjacent ribs. Pleural roughening and tags were seen infrequently. Liver and spleen were enlarged and congested. Placenta from aborted cows and buffaloes had severe congested placenta with pale white foci (Fig 2A).

Histopathological findings: Histopathological examination revealed two types of pneumonia were observed in the lung, suppurative Bronchopneumonia (22 cows fetus and 14 buffaloes fetus) and interstitial pneumonia (13cows fetus and 8 buffaloes fetus). In fetuses with severe suppurative bronchopneumonia, moderate to large amounts of fibrin, cellular debris, macrophages and neutrophils were observed in exudates in the bronchioles and alveoli (Fig.2B). Degenerative changes were also evident in the mucosa of the bronchi or bronchioles of these fetuses. Smaller airways have macrophages and cell debris. Vascular hyperemia and perivascular accumulations of macrophages and neutrophils were the other microscopic findings. In some fetuses, there was amniotic debris. While the lungs with interstitial pneumonia were characterized by severe lymphocytic infiltration into the interalveolar septa with edema these changes tended to have a multifocal distribution. (Fig. 2C).

Livers of aborted fetuses showed diffuse reticuloendothelial cell hypertrophy. All of the affected fetuses had randomly scattered mild to moderate, periportal, mononuclearcell and/ or neutrophils infiltration (Fig.2D). intrasinusoidal granulomatous nodules Mild to severe hydropic degeneration in the hepatic parenchyma were also showed.

The pathological finding in the spleen of infected fetuses were slight lymphoid depletion of the white pulp and mild neutrophils infiltration (Fig.2E). These changes accompanied by diffuse and multifocal reticuloendothelial hyperplasia and mild lymphoid hyperplasia circumscribing splenic vessels. In some cases intra-and subcapsular mononuclear leukocyte infiltration. Small irregular area of necrosis in the splenic red pulp were seen spleen.

Histopathological examination of the placenta of aborted bovine showed necrotic placentitis, characterized by superficial to deep necrosis of the carunculae, associated with haemorrhage, neutrophilic exudates, intralesional and retained fetal tissues in the caruncularcrypts (Fig.2F) in addition large multiple area of dystrophic calcification.

Immunohistochemical findings: Regarding to the immunohistochemical examination, the positive Immunoperoxidase staining of *Brucellamelitensis* antigens were showed as brown, finely granular intracytoplasmic staining 54 positive samples and out of 57from positive animals using CFT.

In our study, strong positive reaction (+++ve) were located in the cytoplasm of macrophages in the cellular debris of alveoli and bronchi of the fetal lung, and in placenta both extracellular in necrotic areas and intracellular within macrophages, neutrophils and trophoblastic cells. Moderate positive reaction (++ve) showed in isolated macrophages in the sinusoids and interstitium in the cytoplasm of macrophages in the red splenic pulp and sinusoids of fetal spleen. While mild positive reaction (+ve) intracellularly the cytoplasm of macrophages and Kupffer cells of the fetal liver, Similarly, the antigens were present within cytoplasm of some hepatocyte (Fig. 3A–3D), biliary duct epithelial cells as well as isolated macrophages in the sinusoids and interstitium. Also, the immunreactivity were found in the cytoplasm of macrophages in the red splenic pulp and sinusoids of fetal spleen. *Brucellamelitensis* antigens were observed in placenta in both extracellular in necrotic areas and intracellular within macrophages, neutrophils and trophoblastic cells.

Ultra-structural findings: TEM of lung showed the cytoplasm of neutrophil and macrophages containing electron dens bodies of cocobacilli were present in the interstitium and located near the proliferating type II alveolar cells (Fig.4A).TEM of Spleen showed neutrophils and macrophages containing moderate aggregations (clusters) of electron dens bodies of intact cocobacilli within the cytoplasm, with partial lysis of its cytoplasm (Fig 4B).TEM of placenta revealed that the Trophoblasts filled with organisms (Fig.4C)were often degenerate; they were swollen and had electron-lucent cytoplasm, lipid droplets, and dilated membranous cisternae devoid of ribosomes, In necrotic infected trophoblastic cells, cisternae were fragmented, and brucellae were free in the cytosol.

Table 2: Results of serological tests for the recognition of brucellosis in aborted cows and buffaloes.

Examined animals	Serological tests							
	RBT		TAT		iELISA		CFT	
	NO	%	NO	%	NO	%	NO	%
cows (N= 48)	**37**	**77.08**	**32**	**66.67**	**35**	**72.92**	**35**	**72.92**
buffaloes (N= 30)	**23**	**76.67**	**23**	**76.67**	**23**	**76.67**	**22**	**73.33**
Total (N= 78)	**60**	**76.92**	**55**	**70.51**	**58**	**74.36**	**57**	**73.08**

Table 3: Bacteriological culture, PCR assay and IHC in aborted fetuses from serologically positive dams

Examined animals	Serological Positive*	Bacteriological culture**	PCR**	IHC***			
				lung	liver	spleen	placenta
cows (N= 48)	**35**	**31**	**33**	**5**	**6**	**11**	**11**
buffaloes (N= 30)	**22**	**21**	**22**	**2**	**4**	**7**	**8**
Total (N= 78)	**57**	**52**	**55**	**7**	**10**	**18**	**19**

*= CFT positive
**=Abmoasal content
***=lung. liver. spleen and placenta
***= lung, liver, spleen and placenta

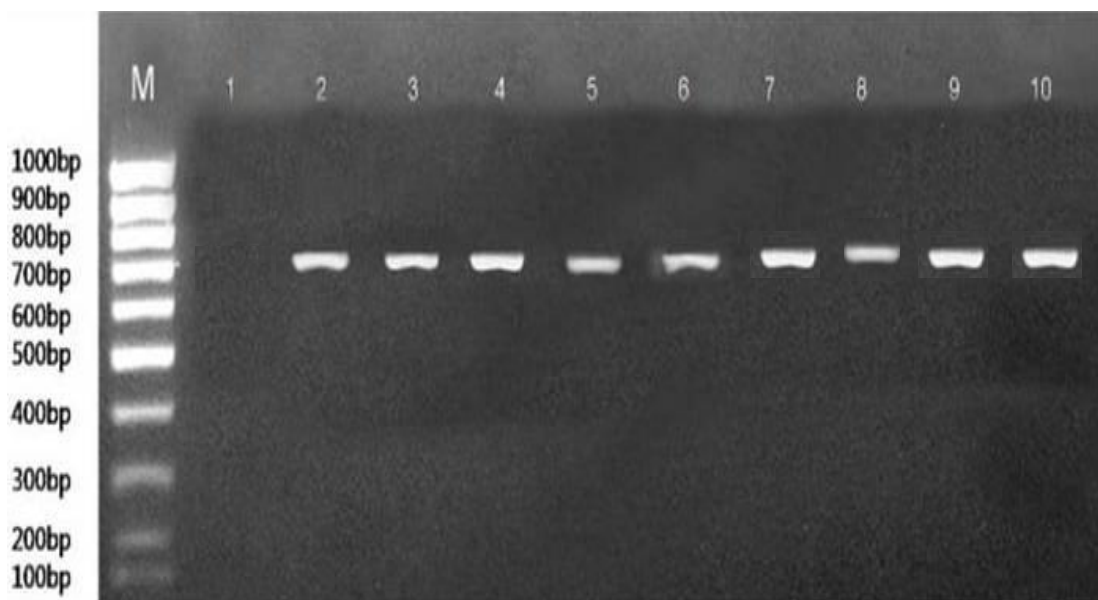

Figure 1: PCR assay for detection of *Brucella* Spp. Lane 1: DNA marker; lane 2: control negative, lane 3: control positive lanes 4–7: positive

Figure 1: lane 1 control negative, lane 2 control positive, lane 3-10 positive samples 731

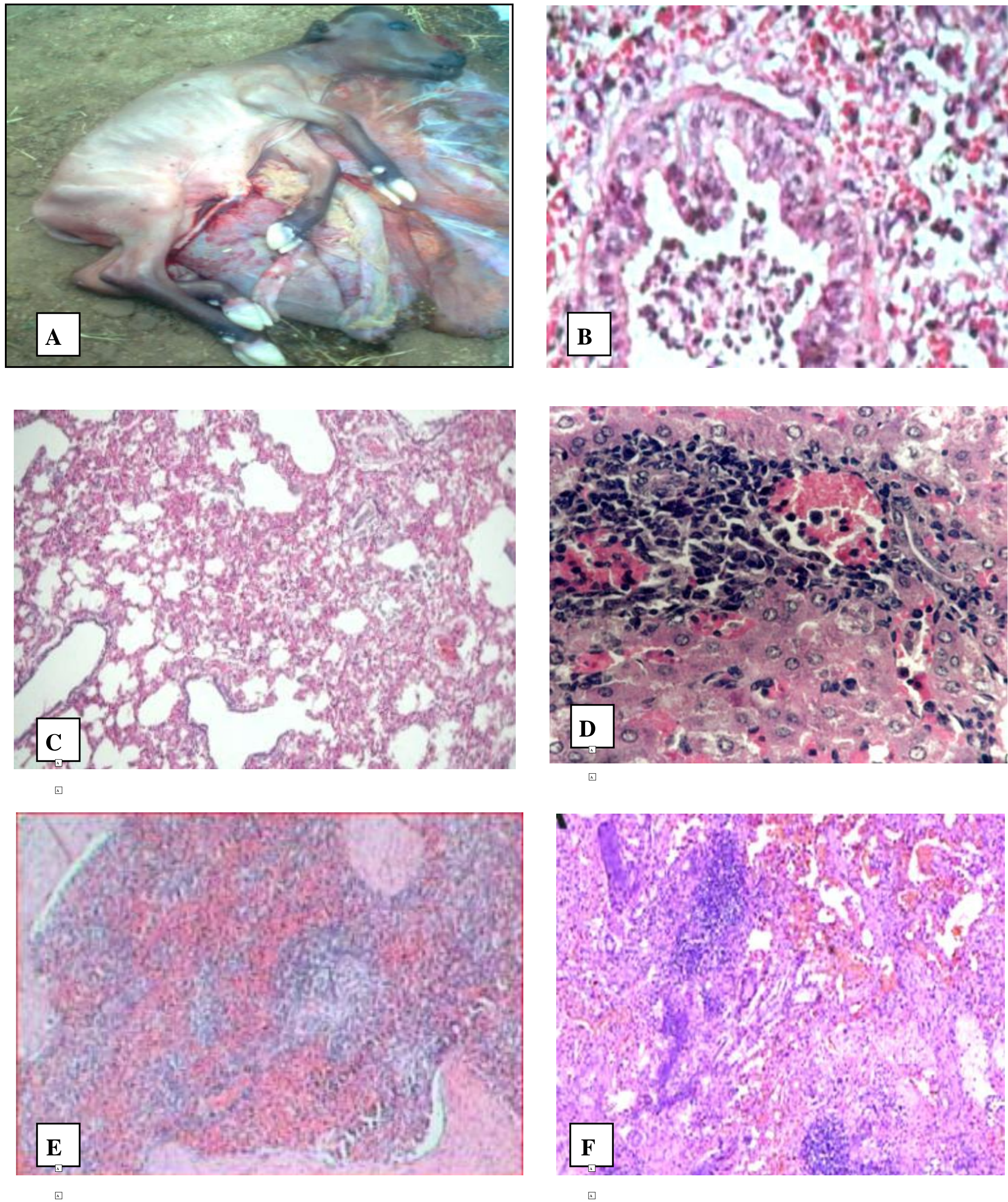

Fig. 2: (A), Aborted fetus due to brucellosis, with edema, opaque and bleeding of placenta fetal. **(B),** lung bronchiole showing inflammatory cell Infiltrate in the lumen and adjacent parenchyma H&E; X200. **(C),** fetal lung showing Interstitial pneumonia with area of alveolar emphysema, the alveolar septa are infiltrated with mononuclear cells (H&E) x100. **(D),** fetal liver showing perivascular polymorphic cell infiltration (H&E; X400.) **(E),** spleen of calf showing lymphoid depletion, mild neutrophil infiltration and hemosiderin granules scattered in splenic parenchyma (H&E;X100). **(F)**, Cow; placenta showing caruncular crypts filled with necrotic debris, multifocal hemorrhage, intense inflammatory infiltrate, (H&E;X100).

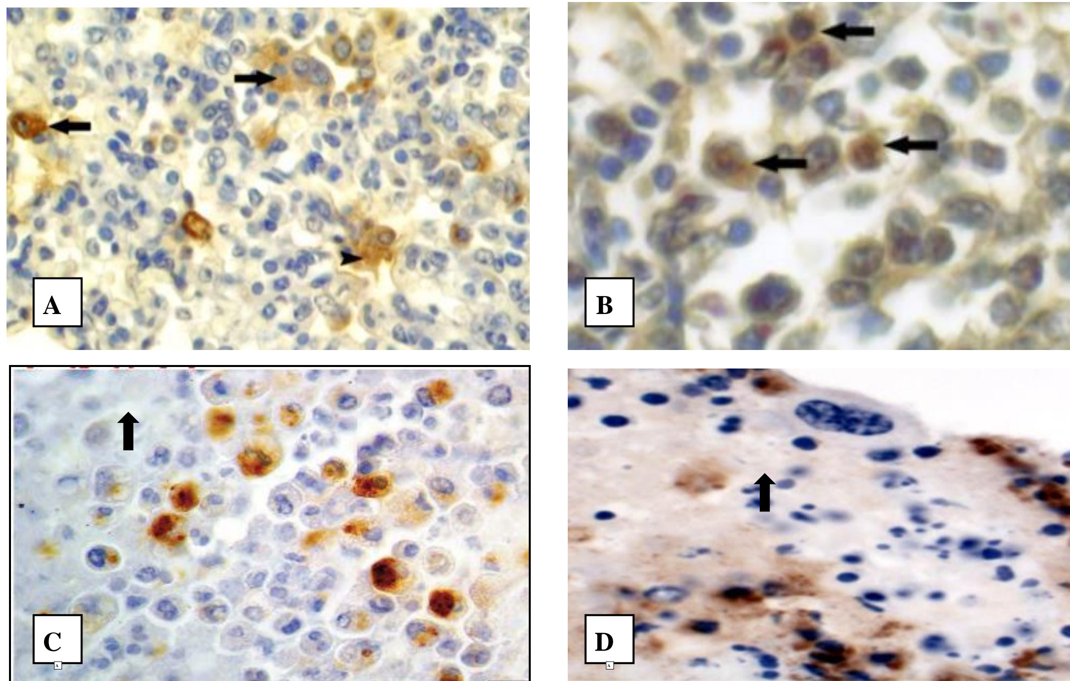

Fig. 3: (A), Photomicrograph of fetal lung showed Immunoreactivity to the anti-*Brucellamelitensis* polyclonal antibody in several macrophages and cellular debris (arrowhead) x 200. **(B),** Photomicrograph of fetal liver showed Immunoreactivity to the anti- Brucellamellitensis polyclonal antibody in the cytoplasm of macrophages and Kupffer cells x 400. **(C)**, Photomicrograph of fetal spleen Immunoreactivity to the anti- Brucellamellitensis polyclonal antibody in the cytoplasm of macrophage of the Red pulp x 400. (**D),** Photomicrograph of placenta showed Chorionic membrane with trophoblastic cells containing intracellular immune *Brucellamelitensis*. x 400.

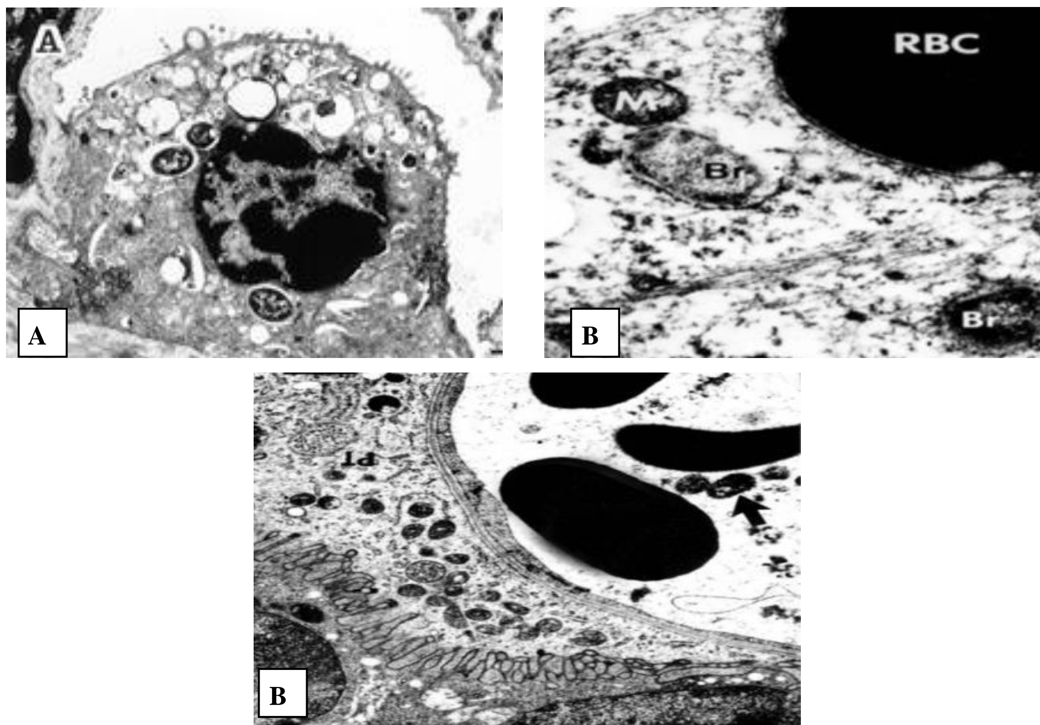

Fig. 4: (A), T.E. Micrograph of fetal lung showed brucella (arrow) in type alveolarepithelial cells X10000 **(B)**, T.E. Micrograph of fetal spleen revealed the presence of moderate aggregates of brucella coco bacilli (Br) in macrophage swelling of mitochondria (M). adjacent erythrocyte (RBC) X10000. **(C)**, T.E. Micrograph of placenta showed *B. melitensis* Filled trophoblasts (arrow), and free brucellae, Sub epithelial capillaries contained intraluminal cocobacilli adjacent erythrocytes (arrow) X10000.

DISCUSSION

Brucellosis is a worldwide zoonotic disease that is recognised as a major cause of heavy economic losses to the livestock industry and poses serious human healthhazard (Ocholi *et al*., 2005). 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 and buffalos 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 nonviable calves and retained placentas (Gabli *et al.*, 2015).

The presumptive diagnosis provided by the serological tests, is usually accepted as indication of brucellosis. Rose Bengal Plate Test (RBPT), Complement Fixation Test (CFT), and Tube agglutination test (TAT) are utilized in this study for the detection of antibodies specific to *Brucella* spp (Pandeya *et al.,* 2013). Serological examination performed by RB test in the present study gave higher number of positive samples 60 (76.92%), as RBT assay can detect antibodies of classes IgG1 and IgM against surface antigen lipopolysaccharides (LPS) of smooth *Brucella* (Davies, 1971). Indeed, this test is internationally acknowledged as the choice for the screening of brucellosis in ruminants (Garin-Bastuji and Blasco, 2004). TAT assay isapproved by the veterinary authority organization in Egypt. However, chronic carriers produce mainly IgG1that block the agglutinating activities of IgG2 (Farina, 1985) which may result in lower detection rates. This mayexplain the lower number of positive samples detected by TAT 55 (70.51%) in comparison to other serological tests used. Excess of antibodies resulting in false negative reaction due to prozone effect (Afify *et al.,* 2013). RBPT provided positive reactors more than TAT, more over due to its ability for earlier detection of recently infected animals as well as the longer persistence of its reaction in those chronically infected as mentioned by (Awad *et al.,* 1977).

In the present study, iELISA provided positive reactors less than RBPT. Similar findings given by (Saravi *et al*., 1995), (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 serologicaltests as it detect all classes of antibodies.

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 crossreaction with other similar gram negative bacteria and so no false results detected Additionally, World Organisation for Animal Health (OIE) suggested that CFT is a test approved all over the world (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 52 aborted bovine (31 cows and 21 buffaloes) out of 57 serologically positive by CFT (91.22%). The studies in various parts of Egypt indicate that the *Br. Melitensis* biovar 3 is the most prevalent field strain (Montasser, 1991 and Afify *et al.,* 2013). The isolation of *Br. Melitensis* strains indicated very high prevalence of *Br. 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).

PCR amplification using published set of primers also resulted in specific amplicon of expectedsize (731 bp) in 33 abomasal content of aborted fetus cow and in 22 abomasal content of aborted fetus buffaloes while no band was observed in negative control (Fig.1). Amplification of microbial DNA from clinical samples offers the potential for rapid, sensitive and specificidentification of pathogens, either directly from tissues or body fluids or after culture of such samples (Gupta *et al.,* 2006). The high incidence of *B. melitensis* in abomasal content samples of unspecific hosts of this present study may show that these animals had been maintained in closeassociation with infected sheep (Kaltungo *et al.,* 2013).

Molecular biology techniques are advancing as a diagnostic tool and will soon be at the point of replacing actualbacterial isolation. These techniques are rapid, safe and cost effective, the only real problems being some uncertainties regarding their specificity. PCR is considered as alternative methods for the failure of culturing and identification of *Brucella* spp. by traditional methods (Samadi *et al*., 2010). Therefore, PCR technique has been revealed to be an important technique for identifying DNA of bacteria and affords a promising alternative method for diagnosis of animal brucellosis.

In this study, *Brucella meletensis* positive aborted bovine fetuses developed histopathologic changes similar to those in experimental and natural infections in cattle (Palmer *et al*., 1996; Perez *et al*., 1998) and buffaloes (Rhyan *et al*., 2001). A series of pathologic

changes in bovine fetuses infected with *Br. melitensis* occur including pneumonia, (López *et al.,* 1984; Hong *et al*., 1991). Bovine fetal pneumonia is the most common lesion and is considered to be diagnostic of *Brucellae* infection by many authors (Meador *et al*., 1989 and Perez *et al*., 1998). In the present study, characteristic pulmonary lesions were bronchopneumonia or interstitial pneumonia which are the most common lesions of aborted fetuses infected with *Br. melitensis*. (Perez *et al*., 1998; Khoudair *et al., 2009*). In this study, in some cases bronchi and bronchioles lumens contained aspirated amniotic fluid and generally considered an important *Brucellae* infection route in affected fetuses (López *et al.,* 1984). Hepatitis is a recognized sequel of chronic brucellosis in humans (Aygen 1998) and other animals (Elzer *et al*., 1998 and Song *et al*., 2008). In the present work, lesions in the liver and spleen typically were diffuse reticuloendothelial hypertrophy, periportal and sinusoidal infiltration of a mixture of lymphocytes with smaller numbers of macrophages. Histological changes described in this report are similar to those recognized in cattle (Perez *et al*., 1998 and Barquero-Calvo *et al*., 2007). As in the present study, mild-to-moderate lymphoid hyperplasia circumscribing splenic arteries and splenic focal necrosis have been described in bovine fetuses (Hong *et al.,* 1991 and Khoudair *et al*., 2009), yet was not observed in caprine and ovine (Yazıcıoglu 1997). It would seem possible that the increased splenic inflammation was in fact manifestation of an effective immune response. It was recently demonstrated in mice that virulent Brucella induced a strong pro-inflammatory response in the spleen, as assessed by evaluating the gene expression profile (Roux *et al*., 2007).

Necrotic neutrophilic placentitis with perivascular infiltrate, which was the most frequently observed microscopical change in experimentally infected bovine, was associated with large numbers of *B.melitensis* intracellularly in macrophages and trophoblasts and also extra cellularly in necrotic tissues. Trophoblasts are thought to be the primary target cell for invasion and multiplication of *Br. melitensis* in the placenta (Anderson *et al*., 1986 and Dey *et al*., 2013). This tropism may be due to the presence of erythritol, or to hormone synthesis by trophoblastic cells (Samartino and Enright, 1993). The specific immunoreactivity which was seen as intense granular staining reaction against the *Br. Melitensis* antigen was detected mainly in the macrophage cytoplasm, in some neutrophils and cellular debris. Regarding to the immunohistochemical examination, the positive Immunoperoxidase staining fo *Brucellamelitensis* antigens were showed as brown, finely granular intracytoplasmic staining 54(33 cows and 21 buffaloes) positive samples out of 57from positive animals using CFT. The higher number of positive IHC werein spleen and placenta. In the present study two fetuses were positive by immunohistochemistry and negative by bacteriologic culture. The reasons possibly due to degenerated microorganisms, deficient isolation technique, or cross reaction of antibody with another antigen (Perez *et al.,* 1998).

An intense antigenic reaction was mainly localized in the cells of the inflammatory foci of the lung, liver, spleen and placenta. However, some isolated inflammatory cells reacted weakly or negative. Similarly, previous reports shown that organisms were located mainly in the cytoplasm of the macrophages in the inflammatory foci (Perez *et al.,* 1998).

In our study, Ultrastructure examination of placenta indicates that *Brucellamelitensis* first enters and replicates within erythrophagocytic trophoblasts. We believe that *Br. melitensis* next replicates in the rough endoplasmic reticulum of chorioallantoic trophoblasts (Fink and Cookson 2005). Chorionic villi and fetal viscera are infected hematogenously after trophoblast necrosis and ulceration of chorioallantoic membranes have occurred. It is likely that *Br. Melitensis* present in placentomal chorionic villi caused vasculitis and separation of trophoblasts from maternal syncytial epithelium. The numerous *Br. Melitensis* present in chorionic connective tissue may be due to failure of fetal phagocytes to destroy *Brucella* and subsequent bacterial replication. Degenerative fetal phagocytes containing intact brucellae were prevalent in chorionic villi (Pei, and Ficht, 2004).

CONCLUSION

Definitive diagnosis of brucellosis remains a difficult task. The only diagnosis, which is the ‘gold standard’, is the isolation of the causative agent from the host. However, it is associated with some problems: low sensitivity, cost and danger due to laboratory infection of personnel. Indirect testing of anti-*Brucella* spp. antibodies in serum, and other clinical specimens are routinely used in brucellosis control and eradication programmes. These tests have, however, been shown to be inconclusive, leading to culling of *Brucella*-free animals and subsequent economic loss. Molecular biology with selected primers as a diagnostic tool is advancing with promising results, and may soon be at the point of replacing actual bacterial isolation. It is rapid, safe and cost-effective, the only real problems, being some uncertainties regarding specificity. The use of immunohistochemistry and electronmicroscopical technique is particularly useful tools for diagnosis of bovine abortion caused by *Brucellamelitensis* specially in suspected cases with negative bacteriologic culture and in cases when serology is not possible.

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دراسات مقارنة للكشف عن ميكروب البروسيلا فى اجنة الماشية المجهضة باستخدام الطرق التقليدية والمناعة الباثولوجية الكيميائية والطرق الجزيئية

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اجريت هذه الدراسة على عدد ٧٨ من الماشية (٤٨ابقار- ٣٠جاموس) استخدمت الأمصال التي تم الحصول عليها للكشف عن الأجسام المضادة لميكروب البروسيلا باستخدام الاختبارات المصلية الروز بنجال اختبار الكارت (RB)، اختبار أنبوب التراص الانبوبى (TAT)، اختبار الاليزا غير المباشر (iELISA) واختبار المثبت المكمل (CFT). تم جمع عدد٧٨ من محتويات المعدة الرابعة من الأجنة المجهضة للعزل البكتريولوجي وتفاعل البلمرة المتسلسل (PCR). كان الهدف من هذه الدراسة تحديد ميكروب البروسيلا فى اجنة الماشية المجهضة باستخدام الطرق التقليدية والمناعة الباثولوجية الكيميائية والطرق الجزيئية. كشفت نتائج التحليل المصلي أن التفاعلات الإيجابية كانت 60 (76.92٪)، 55 (70.51٪)، 58 (74.36٪) و 57 (73.08٪) مع العينات على التوالي. وأظهرت النتائج بواسطةRB وiELISA أعلى إيجابية. وفي الوقت نفسه، تم الحصول على أدنى منها بواسطة TAT,CFT. تم عزل البروسيلا مليتنسيس العترة ٣ من ٣١ من الابقار (محتوى المعدة الرابعة) من الاجنة المجهضة ومن ٢١ من الجاموس (محتوى المعدة الرابعة). تم اجراء تفاعل البلمرة المتسلسل للكشف عن البروسيلا في الأجنة البروسيلا فى اجنة الماشية مجهضة كانت النتيجة ٥٥ عينة ايجابية (٣٣ فى الابقار و٢٢ فى الجاموس) وأظهرت الاختبارات النسيجية المناعية الكيميائية (IHC) بانزيم البيروكسيديز تفاعلات مناعية ايجابية بشكل كبيرردود الفعل في ألاعضاء الداخلية للاجنة المجهضة فى الماشية المصابة بالبروسيلا بشكل كبير في (الرئة والكبد والطحال والمشيمة). وكشفت الدراسة الحالية وجود البروسيلا في أنسجة الرئة والكبد والطحال، والمشيمة من خلال استخدام اختبار الأفيدين البيوتين البيروكسيديز. كما أظهر الفحص باستخدام الميكروسكوب الالكترونى العثور على ميكروب البروسيلا فى الرئة والطحال داخل السيتوبلازم والتي تحتوي على هيئة cocobacilli. داكنة اللون كذلك وجودها فى المشيمة فى كثير من الأحيان. واظهرت الدراسة ان استخدام الاختبارات النسيجية المناعية الكيميائية (IHC) والفحص الإلكتروني للانسجة بشكل خاص هو وسيلة مفيدة مكملة لتشخيص الإجهاض فى الماشية الناجم عن الاصابة بالبروسيلا خاصة في الحالات المشتبه فيها.