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Pathological and molecular studies on Brucellosis in cattle and buffaloes

Dalal H. Hegab¹; Abd El-Baset E. El-Mashad²; Shawky A. Mustafa²; Huda M. Zaki³; Aziza A. Amin²

¹Pathology Department, Animal Health Research Institute

²Pathology Department, Faculty of Veterinary Medicine, Benha University

³Brucella Department, Animal Health Research Institute, Dokki

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ABSTRACT

The samples were collected from 25 uteri of 20 non-pregnant (14 cattle and 6 buffaloes) and 5 pregnant (2 cattle and 3 buffaloes) and 25 supramammary lymph nodes (16 cattle and 9 buffaloes) from abattoirs in El-Menofia governorate, were previously proved to be seropositive. In addition to 6 samples (2 lungs, 2 livers, 2 intestines) were collected from two aborted fetus from *Brucella* seropositive cattle from the farm of Faculty of Veterinary Medicine, Benha University. *Brucella melitensis* was isolated from supra-mammary lymph nodes and was detected in 20 samples (15/16 cattle and 5/9 buffaloes). *B. abortus* was detected in one sample (pregnant buffalo) by Bruce-ladder PCR and whole-genome sequencing. Pathologically, the examined non-pregnant uteri of cattle and buffaloes showed severe degenerative changes in the uterine mucosa which represented by ulcerative endometritis, in addition to fibrosis that was confirmed by Masson's trichrome stain. Focal coagulative necrosis was detected in pregnant cattle uteri and uterine caruncles. Ulceration in the uterine mucosa with mucoid degeneration, and vasculitis were recorded in the uterus of pregnant buffaloes. Meanwhile, lungs of fetus showed fibrinous pneumonia. Extensive degenerative changes were detected in the liver and intestine of fetus. Finally, it was concluded that natural infection with brucellosis causes variable lesions in different organs either in pregnant or non-pregnant animals.

1. INTRODUCTION

Brucellosis is prevalent worldwide in all farm animal species, (Hegazy et al., 2011). The infection was higher in female than males (Sayed et al., 2010). *Brucella* spp. is a Gram negative, facultative intracellular bacterium that is able to survive and replicate in phagocytic and non-phagocytic cells, resulting in a chronic infection in both humans and animals (Franco et al., 2007), allowing further spread of infection (Hosein et al., 2018a)

Test and slaughter policy is applied on *Brucella* infected dairy buffalo farms. Seropositive animals are slaughtered under the supervision of the Egyptian Veterinary Authorities (Hosein et al., 2018a). Isolation of *Brucella* is obtained on culture growth mainly from supra-mammary lymph nodes of seropositive animals from slaughtered house (Salem et al., 2016). *B. melitensis* is isolated from seropositive buffaloes by Bruce ladder multiplex PCR which confirms the presence of genetic material of *B. melitensis* in culture DNA extracts (Hosein et al., 2018b).

Brucella infection in pregnant animals affects the placenta and fetus. Late abortion has been described as the main clinical sign of brucellosis (Martínez et al., 2010). Uterine secretion and products from abortion are the most important source of infection (Neta et al., 2010). The mechanisms of

placenta localization, trophoblast tropism and abortion are poorly understood (Poester, et al., 2013).

Studies on brucellosis have been conducted on aborted fetuses obtained from natural infection (Al-Tememy et al., 2013). The diagnosis of brucellosis is based on the isolation of the pathogen from whole blood, semen, vaginal secretions, urine, and lymphoid tissues (Kim et al., 2010).

Understanding the pathological alterations, the distribution and isolation of *brucella* from the various organs of naturally infected animals and from aborted fetus is important to improve diagnosis and minimize its transmission to humans. Therefore, the present study was designed to isolate and identify *Brucella* species from naturally infected animals, and to demonstrate the *Brucella* antigen in pregnant and non-pregnant cattle and buffaloes using polymerase chain reaction as well as to describe the pathological alterations in the uterus of pregnant and non-pregnant cattle and buffaloes as well as the lung, liver, and intestine collected from the aborted fetuses.

2. MATERIAL AND METHODS

1. History of investigated farm animals:

A total number of 56 samples from *Brucella* seropositive animals. The samples were 25 uteri divided into 20 non-

* Correspondence to: dodomansour71@gmail.com

pregnant (14 cattle, 6 buffaloes) and 5 pregnant (2 cattle, 3 buffaloes), 25 supra-mammary lymph nodes (16 cattle, 9 buffaloes). All these animals were previously identified as *Brucella* seropositive cases by the supervision of the Egyptian Veterinary Services according to discarding culling system to obligatory slaughtered in slaughterhouses in El-Menofia governorate. In addition to 6 samples (2 lungs, 2 livers, 2 intestines) from one aborted fetus and one newborn calf from *Brucella* seropositive cattle from the farm of Faculty of Veterinary Medicine, Benha University. These samples were collected during the period extended from 2018-2019 and employed for pathological and molecular examination for the diagnosis of Brucellosis. Samples were collected and handled with all appropriate precautions. The study was approved by the Ethical Committee of Research at Faculty of Veterinary Medicine, Benha University (approval no: BUFVTM 03-08-22).

2. Samples collection

2.1. Bacteriological and molecular examination:

For bacteriological and molecular examination, supra-mammary lymph nodes were collected from each slaughtered animal. The lymph capsule with the surrounding tissue was excised with taking care to avoid cutting any of the infected surfaces and packed in sterile, separate plastic bags in an ice box at 4 °C and kept in the freezer till examination.

2.1.1. Bacteriological isolation:

Isolation, identification, and bio-typing of *Brucella* organisms were carried out according to the recommendation of the FAO/WHO, Expert Committee on brucellosis. For *Brucella* spp isolation, direct culturing of lymph nodes on selective *Brucella* agar was done and plates were incubated at 37 °C with 5 % CO₂. Media were routinely examined on the 4th day and upwards every 48 hours before being discarded as negative after 3 weeks, the suspected colonies were further identified and sub-cultured on *Brucella* agar and the colonies were identified when plates were held up towards indirect daylight and viewed through the *Brucella* agar media as previously described by Alton et al. (1988).

2.1.2. Biotyping of *Brucella* isolates:

2.1.2.1. Biochemical identification:

The isolates were typed according to CO₂ requirement, H₂S production, Oxidase, Catalase, Urease tests and Gram reaction according to Alton et al. (1988).

2.1.2.2. Matrix-assisted laser desorption/ionization (MALDI TOF MS) based species identification

The microbial species identification was carried out using MALDI TOF MS as described elsewhere by Murugaiyan et al. (2014).

2.2. Molecular detection of *Brucella* spp:

Application of conventional PCR (cPCR) and AMOS PCR. *Brucella* isolates were subjected to DNA extraction from Biomasses using the QIAamp DNA Mini Kit (catalogue number 51306) according to the manufacturer's guidelines. *Brucella* species were identified from the extracted DNA by multiplex PCR according to Alton et al. (1988).

AMOS PCR: AMOS-PCR was performed at the OIE/NRL for brucellosis at Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Institute of Bacterial Infections and Zoonoses, Jena, Germany. Following inactivation of bacteria at 80 °C for 2 hours. DNA was extracted with the high pure PCR template preparation kit

(Roche Applied Sciences, Mannheim, Germany) according to the manufacture instructions. The AMOS PCR (*B. abortus*, *B. melitensis*, *B. Ovis* and *B. suis* PCR) was performed as described before (Bricker and Halling, 1994).

Multiplex PCR: Preparation of multiplex PCR Master Mix according to Emerald Amp GT PCR master mix (Takara) Code No. RR310Akit. 12.5 µl Genaxxon Red Master Mix. the prepared Master Mix of DNA-Polymerase, buffer, dNTP's and loading dye for the gel (1,2% agarose with ethidium bromide), 3 µl of the primer mix, 1 µl DNA, and 8.5 µl water.

Table 1 Used primers for the multiplex Bruce-ladder-PCR

primer	Sequence 5'-3'	Amplificate (bp)	Concentration In primer mix
BMEI0998f	ATC CTA TTG CCC CGA TAA GG	1,682	4,0 pmol 4,0 pmol
BMEI0997r	GCT TCG CAT TTT CAC TGT AGC		
BMEI0535f	GCG CAT TCT TCG GTT ATG AA	450 (1,320**)	1,0 pmol 1,0 pmol
BMEI0536r	CGC AGG CGA AAA CAG CTA TAA		
BMEI0843f	TTT ACA CAG GCA ATC CAG CA	1,071	1,5 pmol 1,5 pmol
BMEI0844r	GCG TCC AGT TGT TGT TGA TG		
BMEI0721f	CCA ACC GTA TGT CCT CTC T	766	1,5 pmol 1,5 pmol
BMEI0722r	TGC GGG AAC TGG TGT TCG ACG		
BMEI0428f	GCC GCT ATT ATG TGG ACT GG	587	1,0 pmol 1,0 pmol
MBEII0428r	AAT GAC TTC ACG GTC GTT CG		
BMEI02f	CTA CTC AAG GAC AAC AGG TG	344	3,0 pmol 3,0 pmol
BMEI02r	TGT GTC GTT TAA GGC AAT AGG		
BR0953f	GGA ACA CTA CGC CAC CTT GT	272	1,5 pmol 1,5 pmol
BR0953r	GAT GGA GCA AAC GCT GAA G		
BMEI0752f	CAG GCA AAC CCT CAG AAG C	218	2,0 pmol 2,0 pmol
BMEI0752r	GAT GTG GTA ACG CAC ACC AA		
BMEI0987f	CGC AGA CAG TGA CCA TCA AA	152	3,0 pmol 3,0 pmol
BMEI0987r	GTA TTC AGC CCC CGT TAC CT		

2.3. Histopathological examination:

Tissue specimens (uterus) were collected from pregnant and non-pregnant female cattle and buffaloes' carcasses after slaughtering in abattoirs. as well as lungs, liver, and intestines from aborted fetus. The formalin fixed specimens were processed by washing, dehydration in ascending grades of ethyl alcohol, clearing in xylene and embedding in paraffin wax. Serial sections, of 3-5 µ thickness were obtained then stained with hematoxylin and eosin stain (H & E) (Bancroft and Gamble, 2002). In addition, Masson's trichrome stain was used to demonstrate the presence of collagen fibers (Bancroft and Gamble, 2008).

3. RESULTS

3.1. Bacteriological results

Brucella melitensis was isolated from 21 out of 25 seropositive animals (15 cattle, 6 buffaloes). *Brucella* cultures showed typical characteristics for the genus *Brucella*. Colonies were smooth elevated, transparent, and convex, with intact borders, brilliant surface and have a honey color under transmitted light. Typing of *brucella* isolates recovered in this study revealed *Brucella melitensis*. Additionally, the colonies of positive isolation samples were picked up on broth for differentiation of *Brucella* species by using PCR assay, after transferring one colony on non-selective media (Nutrient agar) and incubated at 37 °C for

24 hours however, one cattle and three buffaloes were negative for isolation.

3.2. Results of molecular identification

The positive 21 isolation samples were confirmed by Bruce-ladder PCR. From these samples, *B. melitensis* was detected in 20 samples (15/16 cattle, 5/9 buffaloes) and *B. abortus* was detected in one sample from pregnant buffalo.

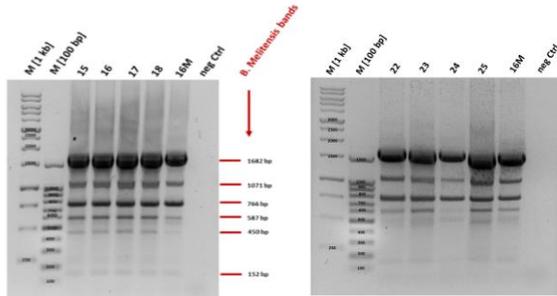


Fig. 1&2: *Brucella melitensis* PCR from culture. Lane M (1 kb). Molecular weight marker (250-3000 bp). Lane M (100 bp): Molecular weight marker (100-1000 bp). Lanes 15-18 in Fig 1 and Lanes 22-25 in Fig 2: samples tested positive for *B. melitensis* (lanes 15,16,17, 22, 23, 25 was for non-pregnant cattle, lane 18 was for pregnant buffaloes' sample but lane 24 was for pregnant cattle sample). Lane 16 M: positive control *B. melitensis*. Lane Neg Ctrl: Negative control.

Table 2 Differences in the rate of brucellosis based on bacteriological isolation and PCR.

Species	Sample number	Isolation		PCR		
		+ve	Rate (%)	+ve	Rate (%)	
Cattle	16	<i>B. melitensis</i>	15	93.75	15	93.75
		<i>B. abortus</i>	-	-	-	-
Buffaloes	9	<i>B. melitensis</i>	6	66.66	5	55.55
		<i>B. abortus</i>	-	-	1	11.11

3.3. Pathological findings:

The microscopical examination of non-pregnant uteri of cattle naturally infected with brucella showed severe degree of degenerative changes in the form of vacuolation and hydropic degeneration in lining epithelial of uterine mucosa (fig. 1a), as well as desquamation of the lining epithelium of the uterine mucosa with diffuse submucosal hemorrhage (fig.1b) and heavy mononuclear leucocytic cellular infiltrations in the lamina propria. Additionally, accumulation of edematous fluid admixed with mononuclear leucocytic cells in the uterine submucosa was recorded (fig. 1c). Marked degree of the degeneration of epithelial cell lining of the uterine glands with heavy mononuclear leucocytic infiltrations (fig. 1d), atrophy and complete loss of their epithelial cell lining in association with its atrophy were found (fig. 1e). Moreover, cystic dilatation of the uterine glands with peri-glandular fibrosis were detected (fig. 1f). Diffuse sub-mucosal fibrosis was demonstrated by Masson's trichrome stain (fig. 1g). Vacuolation of uterine muscles with edema admixed with leucocytic cellular infiltrations was seen. Interestingly, severe thickening and hyalinization of uterine blood vessels together with vasculitis manifested by a proliferation of endothelial cell lining with hyalinization and vacuolation in their walls in combination with leucocytic cellular infiltration was noticed.

The histopathological examination of uteri of non-pregnant buffaloes infected with brucellosis showed severe degenerative changes in the uterine mucosa which was represented by cytoplasmic vacuolation of epithelial cells of the uterine mucosa with mononuclear leucocytic cellular aggregation (fig. 2a). Ulcerative endometritis characterized by entire loss of the uterine mucosa with a breakdown of its basement membrane, submucosal edema with mild mononuclear cellular infiltration was found (fig. 2b). Moreover, mild degenerative changes of the epithelial cells

of the uterine glands with peri-glandular fibrosis (fig. 2c) which taken positive result by Masson's trichrome stain were recorded (fig. 2d). Diffuse fibrosis in the submucosa, as well as perivascular fibrosis, was demonstrated by Masson's trichrome stain (fig. 2e-f).

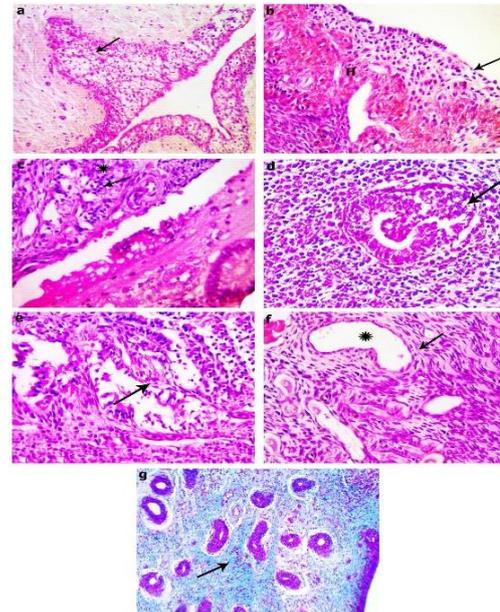


Figure 1 H&E-stained section of the uterus of non-pregnant cattle naturally infected with *Brucella* shows (a) hydropic degeneration of the epithelial cell lining the uterine mucosa (arrow, x400), (b) focal desquamation of the lining epithelium of the uterine mucosa (arrow) with submucosal hemorrhage (H, x400), (c) accumulation of edematous fluid (asterisk) with leucocytic cellular infiltration in the uterine submucosa (arrow, x400), (d) degeneration of the lining epithelium of the uterine gland (arrow) with mononuclear leucocytic cellular infiltration (x400), (e) extensive degeneration and desquamation of the lining epithelium of the uterine gland (arrow, x400), (f) cystic dilatation of the uterine gland (asterisk) with peri-glandular fibrosis (arrow x400), (g) diffuse submucosal fibrosis (Masson's trichrome stain, x100).

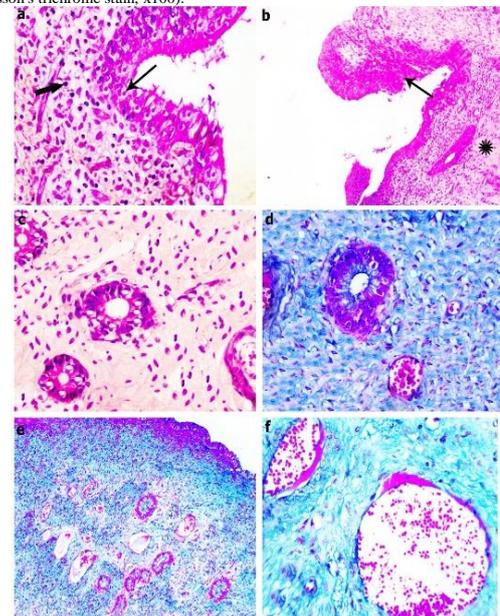


Figure 2 Stained section of the uterus of non-pregnant buffaloes naturally infected with *Brucella* shows (a) cytoplasmic vacuolation of the epithelial cell lining the uterine mucosa (arrow) with mononuclear leucocytic cellular aggregation (thick arrow, H&E, x400), (b) ulcerative endometritis with entire desquamation and necrosis of the lining epithelium of the uterine mucosa (arrow) with submucosal edema (asterisk, H&E, x100), (c) vacuolation of epithelial cells of uterine glands (H&E, x400), (d) peri-glandular fibrosis (Masson's trichrome stain, x400) (x400), (e) diffuse submucosal fibrosis (Masson's trichrome stain, x100), (f) perivascular fibrosis (Masson's trichrome stain, x400).

The microscopical examination of the gravid uterus of the cattle infected with brucellosis revealed severe desquamation of the epithelial cell lining the uterine mucosa with focal areas of coagulative necrosis (fig. 3a-3b), with

diffuse submucosal edema mixed with few inflammatory cells (fig. 3c) Coagulative necrosis of the uterine caruncles. Meanwhile, the uterine submucosa of such cases showed degenerative changes in epithelial cell lining the uterine glands with peri-ductal hemorrhage. Hyaline degeneration of the myometrium was recorded (fig. 3d). Meanwhile, the uterine mucosa of pregnant buffaloes infected with brucellosis showed severe degree of degenerative changes in form of vacuolar and hydropic degeneration of the epithelial cells lining the uterine mucosa (fig. 3e) in association with extensive clear vacuolation of the epithelial cells lining of uterine mucosa with mucoid degeneration of the uterine submucosa (fig. 3f) Marked desquamation and even ulceration of the epithelial cell lining the uterine mucosa were also recorded (fig. 3f). Moreover, vasculitis that characterized by hyperplasia of lining endothelial cells with leukocytic infiltration in the wall of uterine blood vessels.

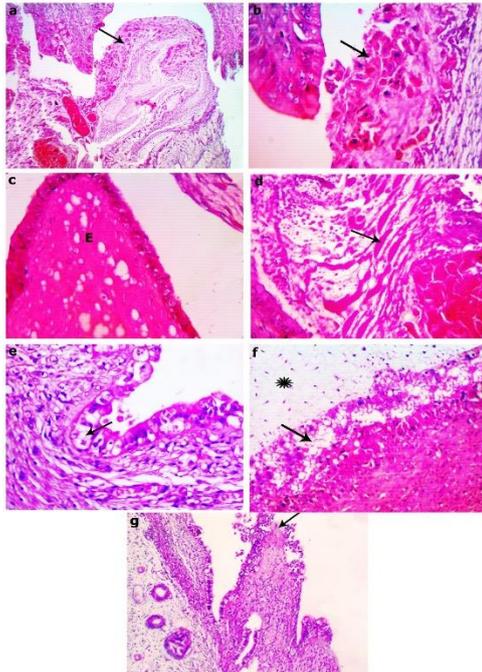


Figure 3 H&E-stained sections of the uterus of pregnant cattle (a-d) and pregnant buffaloes (e-g) naturally infected with *Brucella* showing (a) severe desquamation of the epithelial cell lining the uterine mucosa (arrow, x100), (b) necrosis of the lining epithelium of the uterine mucosa (arrow, x400), (c) accumulation of edematous fluid (E, x400), (d) hyalinosis of the uterine muscles (arrow, x400), (e) vacuolar and hydropic degeneration of the lining epithelium of the uterine mucosa (arrow, x400), (f) extensive clear vacuolation of the epithelial cells lining the uterine mucosa with mucoid degeneration of the uterine submucosa (asterisk, x400), (g) focal ulceration of uterine mucosa (arrow, x100).

The most prominent gross lesion of the lung of the aborted fetus of brucellosis infected cattle showed fibrinous pneumonia as the lung appeared firm, consolidated, dark red color areas, and hepatized in the cut section (fig. 4a). The microscopical examination of the lungs revealed a typical picture of fibrinous pneumonia, as severe desquamation of the epithelial cell lining the bronchioles, with fibrinous exudate in their lumina and the pulmonary alveoli were distended with fibrin (fig. 4b-c) as well as severe congestion and perivascular hemorrhage. Moreover, multiple areas of emphysema were recorded in the neighboring affected areas (fig. 4d). However, the gross appearance of the liver of the aborted fetus showed pale coloration. While, the histopathological examination of the liver of the aborted fetus revealed degenerative changes in the form of diffuse vacuolation of cytoplasm of hepatocyte (fig. 4e), in combination with focal areas of mononuclear leukocytic cellular aggregation (fig. 4f). Accidentally, heavy mononuclear leukocytic cellular infiltration was observed in the portal area. In the meantime, the examined intestine of

the aborted fetus showed a picture of catarrhal enteritis with marked congestion, severe desquamation of the epithelial cell lining the intestinal villi with mononuclear leukocytic cellular infiltration (fig. 4g).

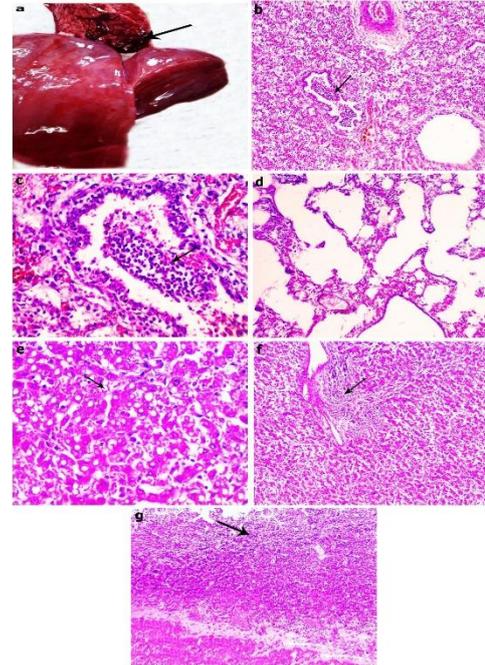


Figure 4 H&E stained sections obtained from the lung (b-d), liver (e-f), intestine (g) of the aborted fetus of pregnant cattle naturally infected with *Brucella* show (a) the lung showing firm, consolidated, hepatized dark red color areas in cut section, (b) fibrinous pneumonia (arrow, x100), (c) distension of bronchioles (arrow), and alveoli with desquamated epithelial cell admixed with leukocytes and fibrin threads (x400), (d) multiple areas of pulmonary emphysema (x400), (e) degenerative changes of hepatocytes in the form of cytoplasmic vacuolation (arrow, x400), (f) focal area of mononuclear leukocytic cellular aggregation in the hepatic parenchyma (arrow, x100), (g) catarrhal enteritis characterized by severe desquamation of the epithelial cells lining the intestine villi with leukocytic cellular infiltration (arrow, x100).

4. DISCUSSION

Bovine brucellosis is a widespread and economically important infectious disease of animals and humans caused by the members of the genus *Brucella*. The disease is manifested by reproductive disorders including storm abortion during the last third of pregnancy, infertility, retained placenta, stillbirth, and low productivity in animals (Refai, 2003). The precise and prompt diagnosis is important for controlling and eradicating the disease. Therefore, the present study carried out to isolate and identify the *Brucella* species from lymph nodes as well as demonstration of the histopathological alterations in uterus of pregnant and non-pregnant cattle and buffaloes as well as lung, liver, and intestine of the aborted fetuses.

In the present study, *brucella melitensis* was isolated from 21 out of 25 seropositive animals (15/16 cattle, 6/9 buffaloes) from supra-mammary lymph node. Isolates of *Brucella* on culture growth mainly from supramammary lymph nodes of seropositive animals (Amin et al., 2012; Salem et al., 2016). *Brucella* cultures showed typical characteristics for the genus *Brucella*. This result agrees with Affi et al. (2011).

The negative isolation in our study (1cattle, 3 buffaloes) may be due to contamination with a low number or non-viable *brucella* organism, the presence of competing organisms (Das et al., 2010) or low bacterial loads in specimens (Godfried et al., 2011). Positive isolation of 21 samples were confirmed by Bruce-ladder PCR. *B. melitensis* detected in 20 samples (15/16 cattle, 5/9 buffaloes), and *B. abortus* was detected in one sample obtained from pregnant buffaloes. These findings were similar with the results of Hosein et al.

(2018b), who used universal PCR-Bruce ladder multiplex PCR that confirmed the presence of genetic material of *B. melitensis* on species level in culture DNA extracts. The most prominent type of brucella was detected in the current study was *B. melitensis* that similar to the findings of Salem et al. (2016).

In our study, the histopathological findings were evident in all seropositive cows as various degree of degenerative changes in different organs were demonstrated due to the colonization by *B. melitensis*. Generally, such tissue alterations induced by brucella infection may result from indirect mechanisms as activation of host immune response through clarified by Oliveira et al. (2008). The histopathological findings in non-pregnant uterus of cattle infected with brucella revealed severe degree of degenerative changes similar to those recorded by Ahmed et al. (2012). Meanwhile, the uteri of non-pregnant buffaloes that infected with brucellosis in present study showed severe degenerative changes in the uterine mucosa, ulcerative endometritis, submucosal edema with mild mononuclear cellular infiltration. Moreover, mild degenerative changes of the epithelial cells of the uterine glands with peri-glandular fibrosis. This result agrees with the findings of Hosein et al. (2018b).

In the present study, the gravid uterus of the cattle infected with brucellosis showed severe desquamation of epithelial cell lining the uterine mucosa with presence of desquamated epithelium in the lumen that agree with Xavier et al. (2009). Additionally, diffuse submucosal edema with degenerative changes in epithelial cell lining the uterine glands in association with hyalinosis of the uterine muscle, these findings were in agree with Ahmed et al. (2012). The uterine caruncles also suffered from coagulative necrosis in our study. This partial agrees with Alcina et al. (2010), who reported that cow placentome with caruncular crypts filled with necrotic debris, intense inflammatory infiltrate, and several bacterial colonies (acute necrotizing placentitis).

In the current study, severe desquamation and even ulceration in the affected uterine mucosa with vasculitis was noticed in the uterus of pregnant buffaloes. These results were in partial agree with Xavier et al. (2009), who documented that endometritis associated with multifocal ulceration of superficial endometrium with peri glandular and perivascular fibrosis was seen in cattle infected with *B. abortus*.

In natural animal hosts, *B. abortus* infection leads to abortion in cows at the late stages of pregnancy due to placental lesions, which are related to bacterial invasion and intracellular replication in trophoblastic cells (Xavier et al., 2009). In the current research, fibrinous pneumonia was observed in the lung of the cattle's aborted fetus infected with brucellosis. This agrees with Xavier et al., (2009), Antoniassi et al. (2016), while partial agree with Fiorentino et al. (2018), who documented that mild broncho-interstitial pneumonia in the aborted fetuses of animals infected with *B. abortus*.

In the meantime, the macroscopic picture of liver of the aborted fetus showed faint coloration which disagree with Antoniassi et al. (2016), who found white areas interspersed with red areas on the hepatic surface of the aborted fetus. The microscopical examination of the liver of fetus in present study revealed vacuolation of the hepatocytes with heavy mononuclear infiltration. These findings agree with Gyuranecz, et al. (2011), but disagree with Dey et al. (2013), who recorded multifocal small necrotic foci in the liver.

In the present investigation, the examined fetal intestine showed catarrhal enteritis, severe desquamation of the epithelial cell lining of intestine villi, severe congestion of

the submucosal blood vessels and perivascular leucocytes infiltration.

5. CONCLUSION

In conclusion, *B. melitensis* was most prominent type of brucella isolated from supra-mammary lymph nodes while *B. abortus* was detected only in pregnant buffalo with MALDI-TOF and PCR. Various degree of degenerative changes in uterus of either pregnant or non-pregnant cattle and buffaloes with fibrinous pneumonia, catarrhal enteritis and hepatic degeneration were predominant in cattle's aborted fetus infected with brucellosis.

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