Research Article

Immune-Pathological Studies on the Distribution and Localization of Bovine Viral Diarrhea Virus

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Abstract | Bovine viral diarrhea virus (BVDV) causes major economic losses in farms as a result of reproductive disorders and immunosuppression. To identify the pathological alterations and Immunohistochemical localization and distribution of BVDV antigen in the genital organs of persistently infected and acutely infected cows, this study was carried out on genital organs of thirty cows submitted to slaughter houses suffering from reproductive disorders. Pathologically, three mummified fetuses had been extracted from the uterus of examined cows (3/30). The disrupted growing and mature follicles were a distinctive microscopic feature in infected animals while such changes were absent in negative cows. The intact oocytes were clearly pronounced only in the negative animals. The fallopian tubes are free from any pathological lesions in the negative animals. Immunohistochemical, BVDV antigen was detected in ear notch, ovaries, fallopian tubes and uterus of one cow (1/30) (PI animal). Whereas, BVDV antigen was detected only in the genital organs of acutely infected animals (2/30) but ear notch was negative. The positive BVDV antigen was clearly observed in the PI cow in the ovaries (primordial, growing, follicles, follicular fluid, granulosa cells, ovarian stroma and blood vessels), the fallopian tube (covering epithelium, sub mucosa and musculosa) and at the uterus (epithelium, endometrial glands, blood vessels and endometrial stroma). While in the acute cases, the positive BVDV antigen could not be observed in the primordial follicles, blood vessels or endometrial glands.

Keywords | Bovine Viral Diarrhea Virus, Immunohistochemistry, Ovaries, Oviducts, PI cow, Uteri

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INTRODUCTION

Bovine viral diarrhea virus (BVDV) is considered one of the major and important pathogens affecting cattle all over the world (Hou et al., 2019). BVDV is belonging to genus Pestivirus, of the family Flaviviridae; two genotypes of BVDV (type 1&2) could be identified infecting ruminants (Smith et al., 2017). There are two biotypes of BVDV, either cytopathic (cp) or non-cytopathic (ncp) based on their cytopathic effects that are appeared in vitro monolayers; cp caused damage to cell culture in the form of apoptosis but ncp biotype did not (Gamlen et al., 2010). BVDV plays an important role on varieties of morphological signs ranging from mild to severe fatal hemorrhagic syndromes and the most important clinical abnormalities are its reproductive disorders which attributed to its tro-

pism for the genital tissues (Ghazi et al., 2008). Only, ncp BVDV has been incriminated to cross the placental barrier, and infects the fetus (Wernike et al., 2018).

BVDV was initially discovered in 1975 (Amin et al., 2014), but little is known of the tissuedistribution of viral antigens in naturally occurring BVDV infections. Immunohistochemistry of skin biopsies has become a useful andreliable tool for BVDV diagnosis. The presence of BVDV antigen in skin is restricted to persistent infection; this methoddifferentiates it from transient infection (Hilbe et al., 2007). Also, this technique was used to detect the virus distribution in different tissues from persistently infected (PI) or acutely infected animals (Frederiksen et al., 1999). The tissue distribution and cellular localizations of BVDV antigen at genital tissues in Egyptian cattle suffering from PI or acutely infection has received less attention. In recent investigations, ovaries are one of the preferred sites for virus replication and this leads to disruption of follicular development (Grooms et al., 1996).

The aim of the present study was to investigate the pathological alterations of female genitalia of cows infected with BVDV as well as describing the antigen distribution and cellular localization of BVDV in the genital organs of the Egyptian PI and acutely infected female cattle.

MATERIAL AND METHODS

Pathological examination

Tissue samples from female genital system (ovaries, uterus and fallopian tubes) and ear notch were collected from thirty cows (aging from 5 to 9 years) admitted to slaughtered house (Belefia abattoir, Beni- Suef governorate, Egypt) due to infertility problems. The post mortem examination was carried out to detect any pathological abnormalities. The obtained samples were fixed in 10% neutral buffer formalin for 24hrs., processed in a graduated ethanol, cleared in methyl benzoate, embedded in paraffin wax, blocked and tissue sections were done at 5 microns and then stained with Hematoxylin and Eosin (H&E) for histopathological examination according to Bancroft and Gamble (2012).

Immunohistochemistry (peroxidase technique–horseradish peroxidase)

Formalin-fixed tissues from ovaries, fallopian tubes, and uterus and earnotch were processed according to (Bancroft and Gamble, 2012). Sections at 5 µm then mounted on positive-charged microscopic slides and subjected to Antigen retrieval through PT link (Leica, Germany) before manipulation in Automated Immunostainer (Leica, Germany) using rabbit primary antibody which was obtained from (VSVRI, Egypt) with dilution 1:500. The EnVition FLEX Dako Kit (code K8000) was used. The used secondary antibody was horseradish peroxidase (HRP). The reaction was visualized by DAB+ chromogen. Positive tissue (lymph node) control slides were made from BVDV positive cases. Negative control slides were section from previously tested tissue without using primary antibody which replaced by normal rabbit serum reagent. The Mayer's hematoxylin was used as counter stain.

Immunohistochemical results were scored (by IHC color intensity) using the following scoring system for each organ where 0 =no detectible antigen; + = mild, or minimal detectible antigen; ++ = moderate detectible antigen and +++ = severe detectible antigen.

RESULTS

According to Immunohistochemical findings of ear notch, the examined animals were allocated to 3 groups; persistently infected animal (PI) about 3.33%; current (acute) infected animals about 6.67 % and control negative animals about 90% Table (1).

Table 1: The prevalence of BVDV infection in slaughtered Egyptian cows.

Item	Persistent infected animal (s)	Acute or current infected animals (s)	Control negative animals
No. of cases	1	2	27
Ear notch(IHC)	+ve	-ve	-ve
Percent of infection	3.33%	6.66%	90%

Gross pathologic changes

Necropsy finding revealed presence of fetal abnormality; in the form of fetal mummifications in three foeti (3/30 cows) one of them was from the PI animal and the other two cases were belonging to the acute infected animals. Uterus carrying mummified fetus appeared enlarged, edematous and on opening to extract this foeti revealed absence of offensive odor with complete absence of fetal fluids. The first obtained mummified fetus was very hard and dry. It has four legs, arched back (sclerosis of vertebrae and show complete resorbed eyes, remnant of umbilical cord (Fig.1-A), collapsed skull as a result of resorption of the brain tissues. The ear notch of this fetus and hismother was immunohistochemically positive against BVDV antigen. The second mummified fetus was very hard on texture. It has neither legs nor eyes. The skull was very hard and had the pyriform shape (Fig.1-B). This fetus belongs to the acute infected group. The third mummified fetus showed hard texture, absence of fore legs and presence of two hind legs. The skull waspyriform in shape and has only eye fissures without eye balls (Fig.1-C). This fetus also belongs to the acute infected group. The female genital organs which

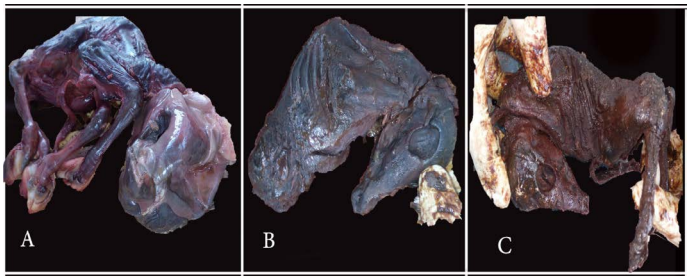

Figure 1: Mummified fetus showing. **A)** Four legs arched back (sclerosis of vertebrae); complete resorbed eyes; remnant of umbilical cord; collapsed skull as a result of resorption of the brain tissues. **B)** Hard on texture; It has neither legs nor eyes; The skull was very hard and had the pyriform shape; This fetus belong to the current or acute infected group. **C)** Hard on texture, absence of one fore legs and presence of two hind legs. The skull was pyriform in shape and has only eye fissures without eye balls, this fetus also belongs to the current infected group.

Table 2: Scoring of BVDV antigen in female genital organs for Persistently and acute infected groups where **0** =no detectible antigen; **+** = mild, or minimal detectible antigen; **++** = moderate detectible antigen and **+++** = severe detectible antigen.

Group /Organ	PI cow	Acute infected cows
Ovary:		
Primordial F.	+++	-
Mature F.	+++	++
Oocyte.	Not present	-
Granulosa cells	+++	++
Follicular F.	++	-
Atretic F.	+++	++
Granulosa C.T.	+++	++
Ovarian stroma.	+++	++
Blood vessels.	+++	-
Uterine Tube:		
Mucosa	++	+++
Sub mucosa.	++	-
Musculosa & serosa.	+	-
Uterus:		
Uterine epith.	+	-
Endometrial glands.	+++	-
Endometrial stroma.	+++	++
Endometriosis.	Not detected	-
Blood vessels.	-	-

examined before sampling did notshow any obvious macroscopic lesions.

The histopathological and immunohistochemical findings

Scoring of BVDV antigen in female genital organs for persistently and acute infected groups where 0 =no detectible antigen; + = mild, or minimal detectible antigen; ++ = moderate detectible antigen and +++ = severe detectible antigen Table (2).

Histopathological and immunohistochemical findings of persistently infected (PI) cow: The examined ovary revealed disrupted primordial follicles, secondary and tertiary follicles and obliterated atretic follicle. Minute focal granulosa cell tumor could be observed in this group. Active corpus luteum with large and small regressing cells together with degenerated and congested blood vessel (Fig.2, A-C).

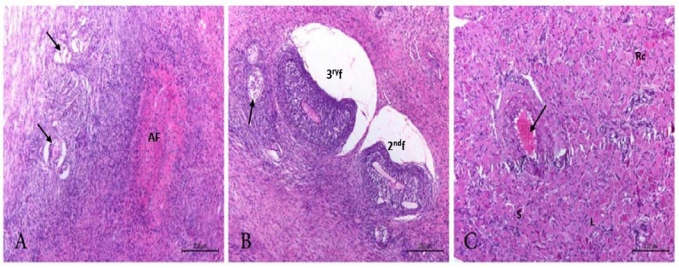

Figure 2: Photomicrograph from ovary of PI cow showing. **A)** Disrupted primordial follicles (**arrow**) and obliterative atretic follicle. (**AF**). H&E. Bar.200µm. **B)** disrupted secondary and tertiary follicles (**2ndf, 3rf**). Minute focal granulosa cell tumor (**arrow**) could be observed. H&E. Bar.200 µm. **C)** Active corpus luteum with large (**L**), small(**S**) regressing cells (**Rc**). The blood vessel showing congestion and degenerated wall (**BVs**) (**arrow**).H&E. Bar.200 µm.

During screening the ovary labeled by immunohistochemistry, the BVDV antigen was clearly detected in follicular fluid. Stromal macrophages were frequently positive. BVDV antigen was also located in the endothelial cells lining stromal blood vessels.

Immunohistochemical investigation of the ear notch revealed positivity against BVDV antigen in the epidermal cells and sebaceous gland (Fig.3. A-D).

The histopathological examination of uterine tube (Infundibulum) from PI animal revealed moderate folding of mucosal layer with congestion, edema and thrombosis of the vasculature. While the Isthmus showed edema and congestion. The IHC staining for uterine tube showed positive reaction as browncolure granules in the cytoplasm of epithelium and mononuclear cells (Fig.4. A-C).

The microscopic examination of the uterus from PI animal revealed necrosis and focal denudation of the endometrial mucosa accompanied with mononuclear cell infiltration mainly lymphocytes and macrophages in the sub mucosa with moderate congestion of the endometrial BVs. Swelling of the muscle fibers of the myometrium with the presence of endometriosis was found.

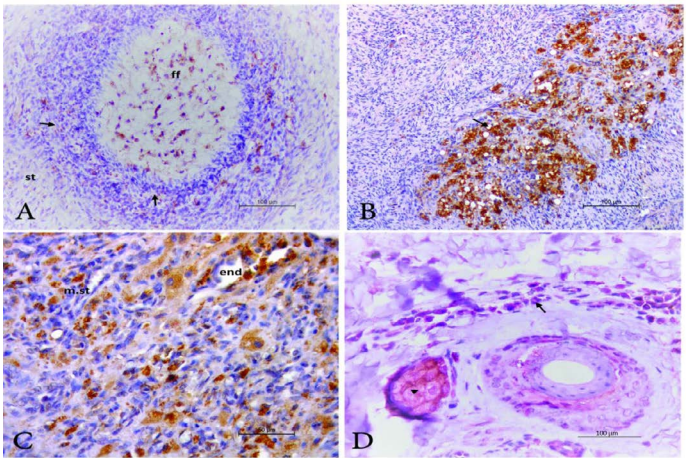

Figure 3: Photomicrograph of ovary and ear notch from PI animal stained by IHC showing, **A)** growing follicles where the positive cells appear at follicular fluid (**ff**); granulosa cells (**arrow**) and at the stromal tissue (**st**). Stained by IHC. Bar.100µm. **B)** BVDV antigen positive cells (macrophages) at the atretic follicles **(arrow**) in the Bar.100µm. **C)** BVDV antigen positive cells (macrophage) in the ovarian medullar stroma (**m.st**) as well as endothelium of the blood vessels (**end**). Bar.50µm. **D)** ear notch revealed positivity against BVDV antigen in dermal cells (**arrow**) and sebaceous gland (**arrow head**); IHC. Bar.100µm.

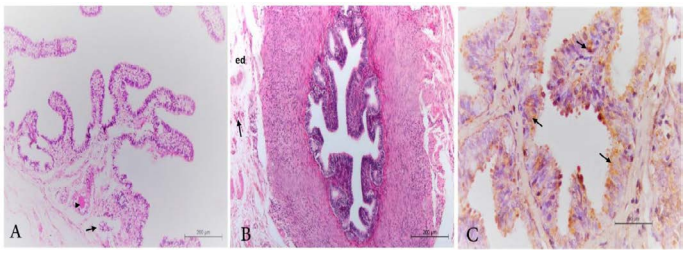

Figure 4: Photomicrograph of uterine tube of PI animal showing. **A)** Infundibulum with moderate folding of mucosa –submucosa layer and congestion **(arrow**) together with edema and thrombosis of the vasculature (**arrow head**) at the tunica serosa and musculosa. H&E. Bar.200µm. **B)** Isthmus suffer from edema (**ed**) and congestion (**arrow**) in stratum vascular. H&E. Bar.200µm. **C)** positive reaction as brown color granules in the cytoplasm of epithelium (**arrow**) IHC labeling. Bar.50µm.

Immunohistochemical labeling of the uterus showed positive BVDV antigen in the endometrial epithelium, macrophages and epithelial cells of the endometrial glands (Fig.5.A-D).

Histopathological and immunohistochemical findings of acutely infected animals: Immunohistochemical investigation of the ear notch revealed negativity against BVDV antigen while it was positive in ovaries, fallopian tubes and uteri.

Pathologically, the common ovarian alterations were follicular atresia with diminished number of primordial follicles, granulosa cell tumors and congestion of medullary blood vessels. Immunohistochemical investigation of these cases clarified positivity against BVDV in the stroma of the ovary, few numbers of the luteal cells, granulosa cells of atretic follicles.

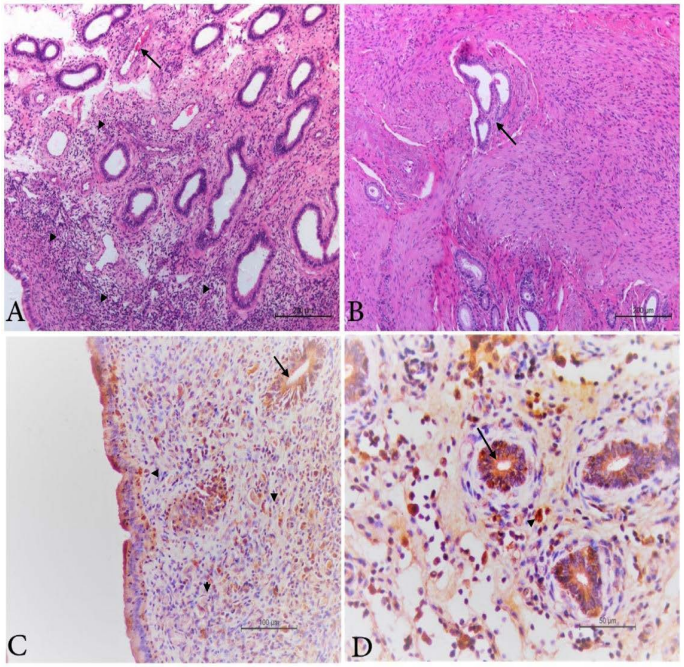

Figure 5: Photomicrograph from uterus of PI animal showing, **A)** endometritis with submucosal and interglandular infiltration with inflammatory cells (**arrow head**) as well as congestion of the vasculature (**arrow).** H&E Bar.200µm. **B)** Islets from endometrial glands between bundles of uterine muscles (endometriosis) (**Arrow**). H&E Bar.200µm. **C)** positive BVDV antigen in the cytoplasm of the glandular epithelium (**arrow**) and in macrophages at sub epithelial, inter -glandular tissue and in the endometrial glands (**arrow head**). IHC labelled. Bar.100µm. **D)** positive brown granules in the cytoplasm of the glandular epithelium (**arrow**) and in macrophages at the inter -glandular tissue (**arrow head**). IHC labelled. Bar.50µm.

Marked folding of mucosal layer and severe congestion of the vasculature were the constant findings in the uterine tube (Infundibulum) from acutely infected animal also edema at the tunica serosa and musculosa could be observed.

The immune-labeled sections revealed positive BVDV antigen in the epithelium and in macrophages at the sub mucosa (Fig. 6.A-G).

The histopathological examination of the uterus revealed acute endometritis in one case and the other manifested feature of chronic endometritis. The acute endometritis

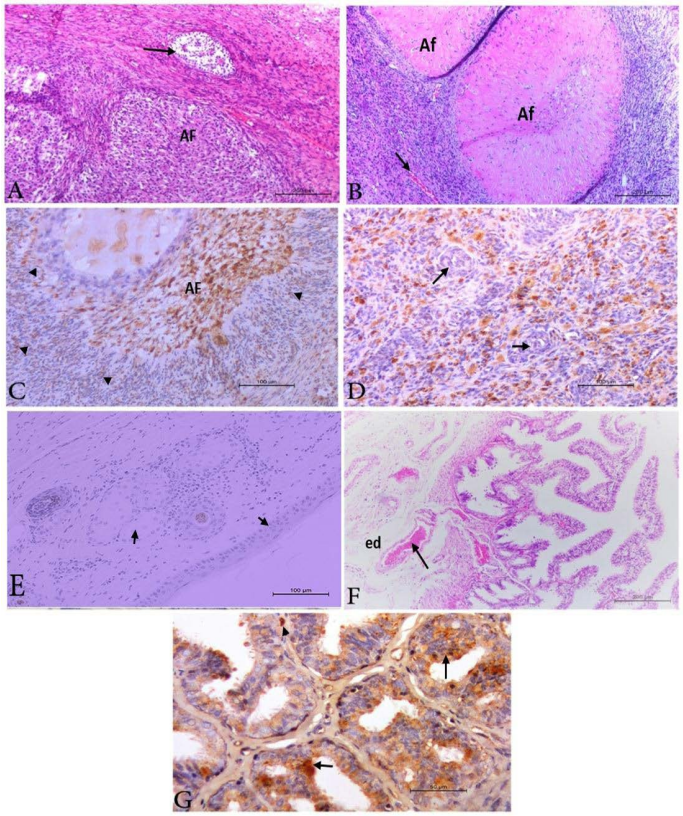

Figure 6: Photomicrograph from ovary, ear notch and uterine tube of acute infected animals. **A)** Ovary with collapsed atretic follicle (**AF**) and focal granulosa cell tumor (**arrow**) .H&E. Bar. 200µm**. B)** Ovary with collapsed atretic follicles (**AF**) and congested stromal blood vessels (**arrow**).H&E. Bar. 200µm**. C)** Ovary revealed positive BVDV antigen in the collapsed atretic follicle (**AF**) and in macrophages at the ovarian stroma. IHC. Bar.100µm. **D)** Ovary with positive macrophages at the ovarian stroma while the blood vessels appear clear negative (**arrow**). H&E. Bar.50 µm. **E)** Ear notch showing negative reaction for BVDV antigen (**arrow**). IHC. Bar.100 µm. **F)** Infundibulum showing marked folding of mucosa –submucosa layer and severe congestion of the vasculature (**arrow**). also edema (**ed**) at the tunica serosa and musculosa. H&E. Bar.200µm. **G)** Uterine tube showing positive BVDV antigen in the epithelium in focal manner (**arrow**) and in macrophages at the sub mucosa (**arrow head**). IHC. Bar.50µm.

had necrosis, sloughing of mucosa, diffuse infiltration of mononuclear cells with congestion and hemorrhages. Hyperplasia of the endometrial glands associated with cystic glandular dilatation in some of which was seen. The chronic endometritis presented by granulation tissue and periglandular fibrosis at the endometrial stroma.

Immunohistochemical examination clarified positivity against BVDV antigen which was detected only in macrophages while the epithelium of endometrial gland was negative (Fig.7, A-D).

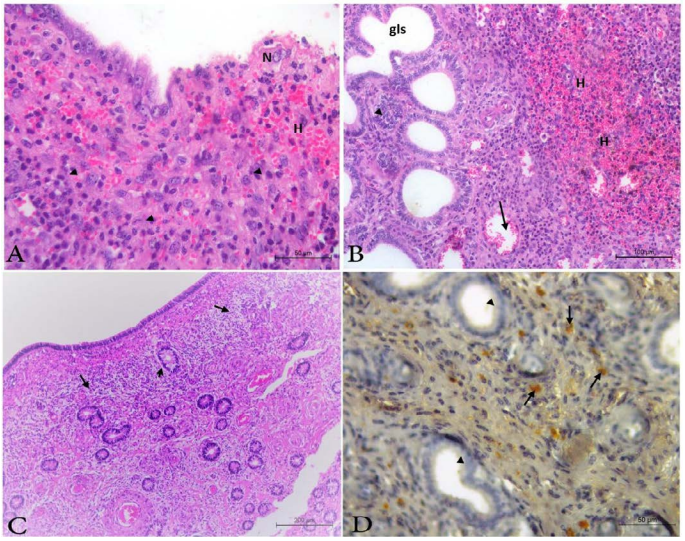

Figure 7: Photomicrograph from uterus of acute infected animals showing, **A)** Necrosis (**N**); sloughing of mucosa; diffuse infiltration of mononuclear cells (**arrow head**); congestion (**arrow**) and hemorrhages (**H**). H&E. Bar.50µm. **B)** Necrosis; hyperplasia of the endometrial gland (**arrow head**) associated with Cystically dilated glands (**gls**) beside congestion (**arrow**) and hemorrhages (**H**). H&E. Bar.100µm. **C)** Chronic endometritis presented by granulation tissue (**arrow**) and periglandular fibrosis at the endometrial stroma (**arrow head**).H&E. Bar.200µm. **D)** brown granules represent the positive BVDV antigen (**arrow**). All uterine glands are free from BVDV antigen (**arrow head**).H&E. Bar.50µm.

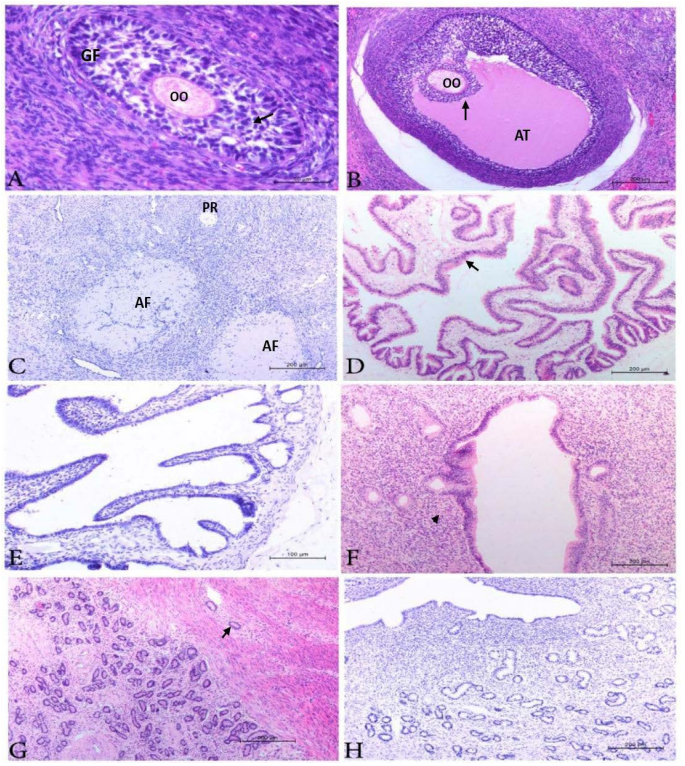

Figure 8: Photomicrograph from ovary, uterine tube and uterus of negative cows showing, **A)** ovary with growing follicles (**GF**) had intact oocyte(**OO**) and corona radiata

(**arrow**). H&E. Bar.50µm. **B)** ovary with mature follicles (**MF**) had intact oocyte (**OO**) and corona radiata (**arrow**) as well as antrum (**AT**). H&E. Bar.200µm. **C)** Ovary with primordial (**PR**) and collapsed atretic follicles (**AF**). No positive reaction could be detected. IHC. Bar.200µm. **D)** uterine tubes showing normal mucosa and sub mucosa. H&E. Bar.200µm. **E)** uterine tubes appear free from BVDV antigen. labelled by IHC Bar.100 µm**. F)** Uterus showing mild chronic endometritis (**arrow head**). H&E. Bar .200 µm. **G)** Uterus with endometrial gland hyperplasia associated with endometriosis (**arrow**). H&E. Bar.200µm. **H)** Uterus showing negative BVDV; antigen. IHC. Bar.200 µm.

Histopathological and immunohistochemical findings of negative animals for BVDV (control group): This group was presented by 27 cows in which both ear notch and genital organs were negative when screened by IHC labeling. Histopathological, the ovaries showed chronic oophritis associated with granulosa cell tumor in three cows. The rest of ovaries were nearly normal with the presence of growing and mature follicles having intact ova. Follicular atresia was also observed. Chronic endometritis was the distinctive microscopic lesion in most cases; which may be associated with endometrial gland hyperplasia, endometriosis and marked periglandular fibrosis. No clear alterations could be observed in microscopic study of fallopian tube (Fig.8, A-H).

DISCUSSION

BVDV considers one of the most common viral infections of cattle; so its control and prevention are of a worldwide concern (Houe, 2003). BVDV infection had a great importance due to its economic losses in farm animals which mainly due to reproductive disorder including infertility, repeat reader, abortion, congenital abnormalities (Lanyon et al., 2014) and immune suppression (Evans et al., 2019). The improvement of IHC technique on formalin-fixed paraffin embedding (FFPE) tissues became easy and highly sensitive with great successes in identifying the PI animals. PI animals should be culled from the herds because it serves as reservoir for BVDV and act as a source of infection. The PI animals shed the virus allover entire their life while the acute infected animals shed the virus to the day 9 post infection (Brodersen, 2014).

The results of the present study revealed three mummified foeti; one of them was from the PI animal and the other two cases were belonging to the acutely infected animals which were calcified and showed deformed head, absence of one or two eyes together with absence of two or four limbs.

Carlsson et al. (1989) explained that BVDV infection leads to corpus luteum lysis meanwhile hormonal imbalance and diminish of progesterone secretion required for maintaining pregnancy and elevation of prostaglandin consequently fetal losses and abortion.

Fetal infection during fetal development and organogenesis in the middle trimester can result in numerous types of congenital anomalies (Lanyon et al., 2014). The combination of direct cellular damage by virus and the resultant inflammatory response to the foreign viral antigens have been proposed as pathogenic mechanisms for congenital anomalies such as cerebellar hypoplasia, microencephaly, hydrocephalus, hydranencephaly, porencephaly and hypomyelination (Otter et al., 2009). Growth retardation, optic neuritis, retinal degeneration, thymic hypoplasia, hypotrichosis, alopecia, curly hair coat, deranged osteogenesis, microopthalmia, cataracts, mandibular brachygnathism were recorded as congenital anomalies accompanied BVDV infection (Espinhasse et al., 1986).

Infection between 80 and 150 days of gestation can lead to fetal teratogenic effects in the fetus. These include cerebellar atrophy, ocular degeneration, brachygnathism (Blanchard et al., 2010), pseudo cyst formation in the brain (Montgomery et al., 2008), and thymus, bone (Webb et al., 2012) and lung growth retardation (Done et al., 1980). Viral infection at this stage can also lead to fetal death and abortion without any effect on the cow (Done et al., 1980).

The histopathological evaluation of ovary from BVD-infected and non-infected cases revealed a few primordial, growing, and mature follicles; some of which had no oocytes and were clearly disrupted in the infected cases, this trend had reversed in none infected animals. Distinctive Inflammation of fallopian tube was observed in infected animals. Acute endometritis was only noticed in acute infected cases. The fallopian tubes are free from any pathological lesions in non- infected animals. The intact oocytes were clearly pronounced only in the negative animals. These findings support idea of (Tsuboi and Imada, 1998) who mentioned that ovary is one of the replication sites of BVDV which lead to abnormal ovum development. They also added that the BVDV was isolated from ovarian follicles from infertile cows. Cow's fertility is reduced due to decreased uterine immunity as a result to BVDV infection (Cheng et al., 2017). Acutely infected animals are responsible for about 93% of uterine infections that results in the birth of PI animals (Wittum et al., 2001).

Therefore, mostly PI animals come from acutely infected dams, but the source of infection of acutely infected animals comes from PI animals (Evans et al., 2019). PI animals originating when the dam and fetus infected with

NCP BVDV during mid gestation (Moennig and Becher, 2018). PI animals act as a hidden source of infection as they are specifically immunotolerant and persistently viraemic and shedding the virus continuously for the long life (Neill, 2013). So, persistently infected animals play the main role in transmission of BVDV than acutely infected animals (Khodakaram-Tafti and Farjanikish, 2017). Among the distribution of BVDV antigen by IHC in the different female genital organs; our results manifested that the positive cells were clearly observed in the PI cow at ovary (primordial, growing, follicles, follicular fluid, granulosa cells, ovarian stroma and blood vessels), fallopian tube (covering epithelium, sub mucosa and musculosa) and at the uterus (epithelium, endometrial glands, blood vessels and stroma). While in the acute cases, the positive BVDV antigen could not be observed in the primordial follicles, blood vessels and endometrial glands or the ear notch. These finding are entirely consistent with the results mentioned by many authors (Shin and Acland 2001; Firat et al., 2002).

Immunohistochemistry is the most useful and reliable tool to study the pathogenesis and diagnose BVDV but also to define the type of the infected cell and localize the antigen within the tissues (Cornish et al., 2005). In PI animals, BVDV was detected in skin, pancreas, udder, lung, liver, kidneys, adrenal glands, brain, digestive tract, and reproductive organs (Njaa et al., 2000; Shin and Acland, 2001; Firat et al., 2002). Macrophages/ histocytes were positive in most cases (Shin and Acland, 2001). In the current study, immunohistochemically, ear notch was positive in only one cow (persistently infected) (3.33%) and was negative in the other 29 animals. But genital organs (ovaries, fallopian tubes and uteri) were positive in both the persistently infected cow and acutely infected animals. In slaughtered cattle, 58.51% of animals were seropositive to BVDV, by 39% in young cattle (Kargar et al., 1995).

Grooms et al. (1998) reported that the follicular growth in the ovaries from acutely infected animals was impaired during the two subsequent estrus cycle after infection. The positivity against BVDV in different developmental stages of growing follicles appeared with varying intensity (Shin and Acland, 2001).

Grooms et al. (1996) observed that BVDV antigens could be easily detected in the luteal cells and macrophage cells in the ovaries of PI animals. In the uterus, BVDV immunostaining reaction has been identified in the endometrial glandular and luminal epithelia, and sometimes, it was present within arterial walls and uterine smooth muscles (Shin and Acland, 2001).

From this study we can conclude that Egyptian cows had the PI and acutely infected cases and the BVDV antigen were distributed in the female genital organs causing intrauterine fetal death and mummification. So we recommend that the veterinary authority should put a program for ear notch IHC testing and culling of PI animals with compensating their owners from animal insurance fund as used in cases of brucellosis. This considers the main step in controlling strategy of this disease.

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CONFLICT OF INTEREST

There is no conflict of interest.

NOVELTY STATEMENT

First research described the localization of BVDV in female genital organ in Egypt.

AUTHORS CONTRIBUTION

All authors participate equally in this research.

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