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Immunohistochemical Detection of Anti-BVDV in Follicular Fluid of She Camel with Respect to the Effect of Infection on *In vitro* Embryo Production and Pathological Structure of the Ovary

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Abstract: The present study is one of the rare studies which aimed to determine the frequency of anti-BVDV in Follicular Fluid (FF) of she camels with assesses risks of BVDV infection on *In vitro* embryo production and histopathological changes in the ovarian structure. Ovaries from freshly slaughtered she camels of unknown breeding history were collected, macroscopically examined and only those having apparently normal-looking follicles (n=46) were selected. From each ovary, FF was aspirated. The selected immature oocytes were matured, fertilized *In vitro* using the standard methodology with some modifications. Anti-BVDV was detected in FF by Immunoperoxidase monolayer assay (IPMA) and Monolayer-enzyme linked immunosorbant assay (M-ELISA). Each ovary was sectioned to 3 portions and preserved in 10% neutral buffered formalin for histopathological examination. Results revealed that, Brown intracytoplasmic granules were detected in 26 out of 46 (56.52%) wells of the inoculated MDBK cellular monolayers. Other 3 samples over the IPMA positive samples were positive by M-ELISA. The pathological changes in the infected ovaries were characteristic for chronic oophoritis represented by presence of mononuclear cells (mostly plasma cells and/or lymphocytes and few histiocytes) along with edema and congested blood vessels in the interstitial tissue of cortical stroma. BVDV infection caused a drastic significance decrease ($P<0.05$) in the fertilization rate as compared to the control group (44.07 versus 23.68%), reduced the cleavage rate, morula and blastocyst stages development (15.22, 4.35 and 0.00%) as compared with the control group (38.89, 22.22 and 13.89% respectively). In conclusion, this work debated the infection of camels with BVDV in Egypt. BVDV infection has great potential effects on ovarian dynamics, structures, IVF and embryo development in she camels. It is appeal with development of IVF and embryo production system in camels, all materials of animal origin must be screened against BVDV contamination. Further studies on other reproductive problems associated with BVDV infection of camels should be interested.

Key words: Anti-BVDV · She camels · Follicular fluid · Immunohistochemistry · IVF · Histopathology

ITRODUCTION

Pestiviral infections have been reported in a wide variety of small and large domestic ruminants, with the best characterized being bovine viral diarrhea virus (BVDV) of cattle, border disease (BDV) of sheep and classical swine fever virus (cSFV) of pigs [1]. Through the implementation of serologic assays, the host range of pestiviruses was noted to include a much broader spectrum, such as antelope, deer and elk [2]. This observation has raised questions regarding the potential for interspecies spread of viruses between domestic livestock and wildlife ungulates [3].

Earlier reports based on serology detected pestiviral (BVDV) antibodies in members of the camelid group, ranging from a low of 4% to a high of 53%. These studies indicated that members of the camelid group are susceptible to infection and do seroconvert. Over the past decade, clinical reports have documented disease conditions in llamas, alpacas and more recently, camels. These conditions ranged from respiratory and enteric diseases to chronic wasting and in utero infections resulting in stillbirths and abortion [4, 5].

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The growing application of *In vitro* embryo production systems that utilize slaughterhouse tissues as a source of both cumulus-oocyte complexes (COCs) and co-culture feeder cells of animals of unknown health status conveys the risk of disease transmission. One pathogen of concern in this regard is BVDV. Therefore, the use of such materials in the *In vitro* fertilization systems represents a potential risk for the transmission of BVDV to embryos and via embryo transfer to the dams [6-9].

In Egypt, without organized BVDV control programs, vaccination is commonly used to control BVDV in cattle only. Serological surveys on BVDV prevalence in cattle population in rural Egypt revealed high incidence (51.84%) of infection [10].

The present study is one of the rare studies which aimed to determine the frequency of anti-BVDV in follicular fluid (FF) of she camels. Assessing risks of BVDV infection on *In vitro* embryo production that utilize slaughterhouse tissues and histopathological changes in the ovarian structure were other targets of this study.

MATERIALS AND METHODS

Ovarian Samples: Ovaries (n=51) from freshly slaughtered she camels of unknown breeding history were collected, transferred to the laboratory, macroscopically examined and only those having apparently normal-looking follicles (n=46) were selected. From each ovary, FF containing immature oocytes was aspirated by 21g needle fitted to 10 ml syringe. Each ovary was sectioned to 3 portions (represented the medial pole, middle portion and lateral pole) and preserved in 10% neutral buffered formalin for histopathological examination.

Virus and Cell Culture: As described by Park *et al.* [11] and Stringfellow *et al.* [12], a reference international BVDV-NADL (National Animal Disease Laboratory) strain was used and proliferated into Madin-Darby bovine kidney (MDBK) cells in minimal essential medium (MEM, Biowhittaker, Walkersville, MD, USA) supplemented with 2% fetal bovine serum (FBS, Biowhittaker, Walkersville, MD, USA) as maintenance media (M-MEM), 200 mM HEPES buffer, sodium bicarbonate (0.75 mg/ml), L-glutamine (0.29 mg/ml) and antibiotics/antimycotic [penicillin G (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml)].

Aliquots of NADL stock virus were quantitated using the method of Reed and Muench [13] prior to initiation of the tests. The cell culture infective dose 50 (50% endpoint $CCID_{50}$) was $10^{6.4}$/ml.

Immunoperoxidase Monolayer Assay (IPMA): The test was performed as developed by Saliki *et al.* [14] and OIE, [15] with some modification to adapt antibodies detection instead of viral detection. Briefly, into 96 well tissue culture (TC) plate, MDBK cells were seeded at a concentration 15 x 10^3 cells/well. In the next day and after 80% of the monolayer sheet was developed, 15 μl of 100 $CCID_{50}$ NADL strain suspension was added to each well and the plate was incubated at the standard culture conditions (37°C, 5% CO_2 and 85% humidity) for one h. The M-MEM was added to the cells after removal the inoculums and the plate was incubated at the standard culture conditions for 48 hrs. The cells were fixed by cold acetone/methyl alcohol (50% v/v) for 20 minutes and dried at room temperature for 3 hrs. The fixed cells were rinsed 3 times by phosphate buffered saline (PBS) pH 7.2. To inhibit endogenous peroxidase, 100 μl/well of freshly prepared 3% H_2O_2 was added for 5 minutes then washed 3 times by PBS. To avoid non specific reaction, two drops/well of a mixture (4% bovine serum albumin; BSA and 1% FBS) were added and the plate was incubated under the standard culture conditions for 15 minutes. The plate was thoroughly washed by the washing buffer (1% tween 80 in PBS) for 3 times, 5 minutes each. Follicular fluid was diluted as 1/8 in predetermined solution (1% tween 80 and 5% horse serum in PBS) and added as 100 μl/well and incubated at 37°C for one h, washed 3 times as above. After that, 100 μl/well of diluted horseradish peroxidase conjugated protein A at a final dilution 10^{-3} in PBS pH 7.2 (BioRad, USA) was added and incubated at 37°C for one h and washed as above for 3 times. The chromogen, Diaminobenzidine tetrahydrocloride (DAB), was prepared as 5 mg of DAB in 10 ml of 0.05 M Tris-HCl pH 7.4 (6.1 g Tris-base, 50 ml deionized water and 37 ml of 1N HCl) and filtered through filter paper before addition of 150 μl of freshly prepared 3% H_2O_2. To each well, 100 μl of chromogen was added and incubated at room temperature for 5 minutes before washing the plate thoroughly by distilled water. Two drops of haematoxylin counter stain were added to each well and rinsed immediately thoroughly under tap water for 15 minutes followed by air dried. The plate was microscopically examined for intracytoplasmic brown granules using an inverted epifluorescence phase-contrast trinuclear microscope (Nikon ECLIPSE-TS100, Japan) with 10X and 20X plan a chromatic lenses and a digital camera DS-U2 with its software NIS elements. Positive and negative controls were included.

Monolayer-Enzyme Linked Immunosorbant Assay (M-ELISA): As outlined by Saliki *et al.* [14], the plates were prepared for M-ELISA as in IPMA with some exception (i): in each plate, one well as blank, 2 wells as negative and 2 wells as positive control were included, (ii): each sample is examined as duplicate, (iii), the soluble chromogen was prepared (0.4 mg O-phenylenediamine (OPD) and 0.4 μl of 30% H_2O_2/ ml of 0.01 M citrate buffer pH 5) and added as 100 μl/well. After the color development, the colored solutions were transferred to another new plates and the reaction was stopped by 50 μl/well of 1/9 sulfuric acid in water, (iiii): the optical density (OD) of the plates was read at 492 nanometer (nm) wavelength, (iiiii): the cutoff end point was calculated by divided OD summation of the negative control and the positive control on the number 4. The samples which their OD > the cutoff endpoint were considered positive, while the samples which their OD ≤ the cutoff endpoint were considered negative.

***In vitro* Maturation and Embryos Production:** In these experiments, oocytes and embryos were produced using the standard methodology as mentioned by Bielanski *et al.* [7] and Holm *et al.* [16] with some modifications. Briefly, COCs were recovered by aspiration of 3 to 6 mm follicles from ovaries of slaughtered she camels and subsequently matured in TCM-199 medium with Earle's salt (Sigma Chemical Company, St. Louis, Missouri, USA) supplemented with 100 μg/ml follicle stimulating hormone (Folltropin; Vetrepharm, Belleville, Ontario), 10 IU/ml human gonadotropin (Chorionic Gonadotropin; Ayerst Veterinary Laboratory, Guelph, Ontario) and 10% FBS at 38°C and 5% CO_2 for 36 hrs. After maturation, some matured oocytes were examined for the signs of maturation and the others were fertilized *In vitro* with epididymal spermatozoa.

Epididymal Spermatozoa Flushing: As carried out by Parrish *et al.* [17], the epididymal sperm suspension was incubated for 10 minutes at 39°C in high humidity (95%) and 5% CO_2. For IVF, the sperm suspension was washed twice with sperm TALP by centrifugation at 250 xg for 10 minutes each time. The pellet was overlaid with fertilization medium (F-TALP supplemented with 4 mg/ml BSA, 50 IU penicillin and 10 μg/ml heparin) and allowed to swim up for 30 minutes in 5% CO_2 incubator. The matured oocytes were washed 3 times in fertilization medium and randomly distributed in 10 mm Ø polystyrene culture dishes (5-10 COCs/drop of 50 μl fertilization medium). The motile spermatozoa were added to the oocytes at a final concentration $2x10^6$/ml. Sperms and oocytes were co-incubated under the same above condition for 24 hrs. Some oocytes were examined for the signs of fertilization and the others were cultured for further *In vitro* embryo development.

***In vitro* Culture:** Following IVF, presumptive zygotes were gently vortexed in PBS supplemented with 50 μg/ml gentamicin to remove spermatozoa or extra cumulus cells still attached to the zygotes. All zygotes were washed 3 times in PBS before being transferred into the culture drops and incubated at 39°C, 5% CO_2 and 95% humidity. The first cleavage (2 to 8 cells) was assessed 48 hrs post-insemination, whereas the development to morula and/or blastocyst stages was assessed on 4-7th day of incubation as performed by Herrler *et al.* [18].

Gross Examination and Histopathology: All ovaries were examined grossly for recording the number of follicles and corpora lutea. Tissue specimens were processed in an automated tissue processor, embedded in paraffin, sectioned at 5μm, stained with hematoxylin and eosin and examined by light microscope as discussed briefly by Bancroft and Gamble [19].

Ovaries of 29 animals that were positive for BVDV infection and those of 17 animals that proved to be negative for BVDV infection were microscopically compared concerning the pathological findings and the absolute number of the histological structures (antral, Graafian, atretic, luteinized follicles and the corpora lutea/corpus albicans/corpus haemorrhagicum) in the ovarian sections.

Statistical Analysis: The proportion of fertilized oocytes, cleaved and those that developed to the morula and blastocyst stages were evaluated by Chi-square test (X^2). A probability of less than 0.05 ($P<0.05$) was considered statistically significant and less than 0.01 ($P<0.01$) was considered statistically highly significant.

RESULTS

Results of IPMA: As illustrated in figures 1A and B, Brown intracytoplasmic granules were detected in 26 out of 46 (56.52%) wells of the inoculated MDBK cellular monolayers with FF while no granules were detected in the other 20 samples.

Results of M-ELISA: The positive samples in which a bright yellow color was developed and their OD > cutoff endpoint were 29/46 (63.04%) that other 3 samples over the IPMA positive samples were positive.

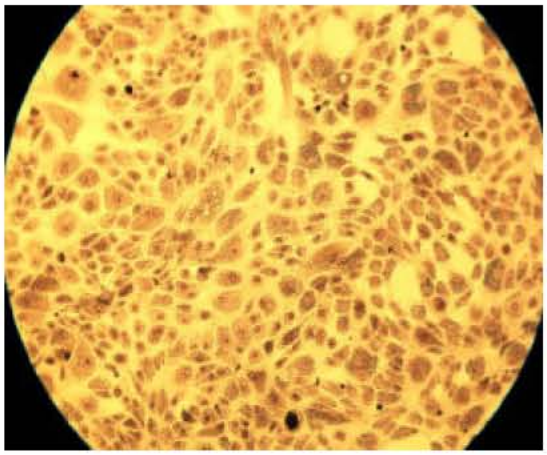
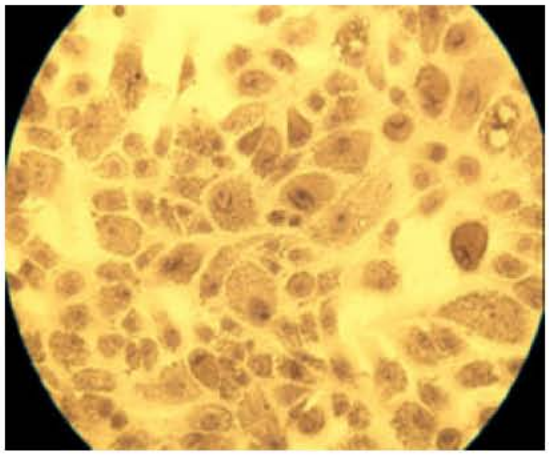
(A)  (B) 

Fig. 1 A and B: Brown intracytoplasmic granules detected of the inoculated MDBK cellular monolayers with FF. (A X 100 and B X 200).

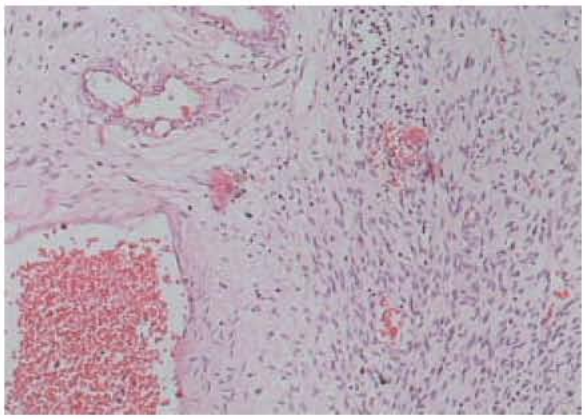


Fig. 2: BVDV infected ovaries showing marked edema, congested blood vessels, and moderate lymphocytic infiltration in the interstitial tissue of the cortical stroma (X 100).

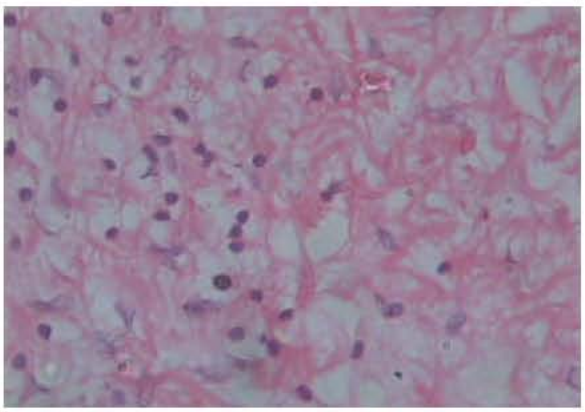


Fig. 3: BVDV infected ovaries showing marked infiltration of the interstitial tissue of the cortical stroma with plasma cells along with marked edema (X 400).

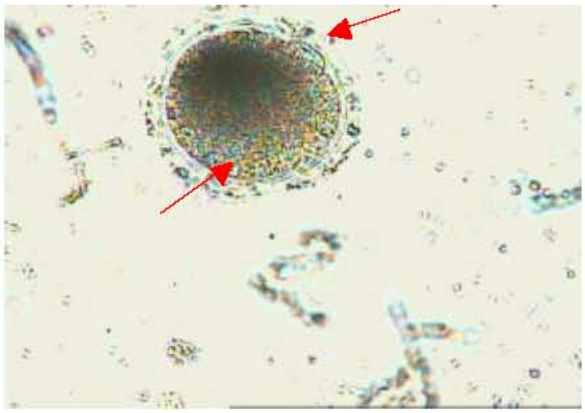


Fig. 4: Microphotograph of fertilized BVDV-free she camel oocyte showing 2nd polar body and sperm tail (x 40)

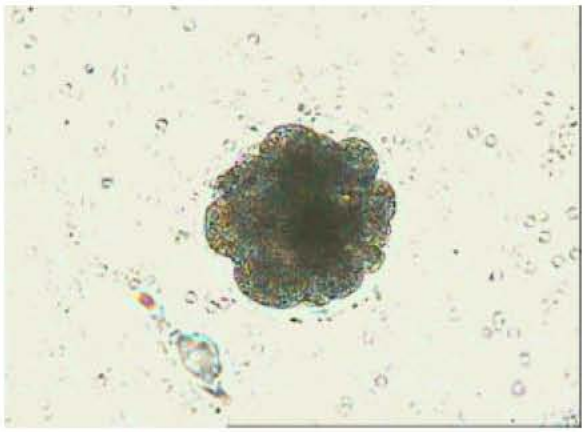


Fig. 5: Microphotograph of BVDV-free she camel oocyte developed *in vitro* to morula stage (x 40)

Table 1: Influence of BVDV infection on *In vitro* penetration and fertilization rates of she camels oocytes

	No. of oocytes	Penetration rate No. %	Fertilization rate No. %	Polyspermy No. %
Control (BVDV free)	59	38 (64.40)[a]	26 (44.07)[a]	6 (10.16)[a]
BVDV (+ve)	76	42 (55.26)[a]	18 (23.68)[b]	20 (26.32)[a]

Values with different letters in the same column were significantly different (P<0.05)

Table 2: Influence of BVDV infections on *In vitro* embryos development

	No. of inseminated oocytes	Cleavage rate No.%	Morula No.%	Blastocyst No. %
Control (BVDV free)	36	14 (38.89)[a]	8 (22.22)[a]	5 (13.89)[a]
BVDV (+ve)	46	7 (15.22)[b]	2 (4.35)[b]	0 (0.00)[b]

Values with different letters in the same column were significantly different (P<0.05)

Pathological Findings: The pathological changes in the ovarian sections in the infected ovaries (29) were characteristic for chronic oophoritis and represented by presence of mononuclear cells (mostly plasma cells and/or lymphocytes and few histiocytes) along with edema and congested blood vessels in the interstitial tissue of cortical stroma (Fig. 2, 3). However, infiltration of the interstitial tissue of cortical stroma with the mononuclear inflammatory cells was ranged from mild to marked or severe. In BVDV-non infected ovaries, 3 had chronic oophoritis, 3 with mild edema in the interstitial tissue of cortical stroma and one with non-luteinized follicular cyst.

The Effect of BVDV on *In vitro* Fertilization Rate and Embryonic Development: As presented in tables 1 and 2, BVDV infection caused a drastic significance decrease (P<0.05) in the fertilization rate as compared to the control group (44.07 versus 23.68%) (Fig. 4). Moreover, BVDV infection drastically reduced the cleavage rate, morula and blastocyst stages development (15.22, 4.35 and 0.00%) as compared with the control group (38.89, 22.22 and 13.89% respectively) (Fig. 5).

DISCUSSION

Pursing literatures of the last decade, the effect of BVDV infection on fertility and reproduction performance received most of scientific attention. Rendering IVF embryos free from contamination with BVDV using the sequential washing procedure recommended by the International Embryo Transfer Society (IETS) is not effective [20, 21].

Bovine viral diarrhea virus was isolated from cells and fluids of ovarian follicles of the cattle [22, 23] and buffalo [24]. On the other hand, immunoglobulins were also found in the fluid of ovarian follicles and many veterinarians rely on BVDV serology which is an important monitoring tool for BVDV infections [9, 25]. Little informations about the pestiviral infection of the reproductive system of she camels are present. The present study is one of the rare studies which aimed to determine the frequency of anti-BVDV in FF of she camels with assesses risks of BVDV infection on *In vitro* embryo production and histopathological changes in the ovarian structure.

There are no pathognomonic clinical signs of infection with BVDV. Diagnostic investigations therefore rely on laboratory-based detection of the virus, or of virus-induced antigens or antibodies in submitted samples [26-28]. Several antigen-capture ELISAs and immunoperoxidase have been described for detection of the BVDV antibodies in serum [29-31]. The IPMA and M-ELISA procedures were designed for persistent infected as well as immunized animals which are known to secrete large amount of BVDV antibodies. Therefore, they are recommended for use in whole-herd screening testing.

Most ovaries in the experimentally as well as in naturally infected cows were palpably smaller and softer than those in non infected ones that the outcome of pestivirus infection depends upon the damage extend to actively dividing cells. Follicular cells and oocytes are permissive to BVDV at all stages of follicular development that namely impairment of oocyte quality and disruption of gonadal steroidogenesis [32-35].

In this study, the pathological changes in the ovarian sections in the infected ovaries (29) were characteristic for chronic oophoritis and represented by presence of mononuclear cells (mostly plasma cells and/or lymphocytes and few histiocytes) along with edema and congested blood vessels in the interstitial tissue of cortical stroma.

Mammalian oocytes and preimplantation-stage embryos are surrounded by a zona pellucida (ZP), glycoprotein cellular matrix. The ZP plays an important role in many physiological functions, including fertilization, block to polyspermy, transport of embryos through the oviduct and containment of blastomeres. Furthermore, the ZP plays a major role as a protective shell against infection of embryonic cells and as a carrier of infectious agents in the spread of livestock diseases through embryo transfer (ET) practices [21, 36].

Extensive experimentation has shown that an intact ZP, both *In vivo* and *In vitro* fertilized oocytes, is an effective barrier against penetration by several animal pathogens, although some viruses and bacteria can bind strongly to it [36]. The mechanism of pathogens binding to ZP is unknown. However, it has been demonstrated that pathogenic agents are more likely to adhere to the surface of ZP of *In vitro* fertilized (IVF) embryos than to that of *In vivo* fertilized embryos [37].

In the present study, BVDV infected ovaries showed drastic significance decrease in the fertilization rate and *In vitro* embryo production as compared with the control group. This is in agreement with Bielanski and Dubuc, [23] who found decreased embryonic development of IVF embryo produced from cows infected *In vivo* with BVDV. This may be attributed to ovarian heat stress (as a result of chronic oophoritis) that hasten *In vitro* maturation of oocytes which lead to ageing of oocytes when *In vitro* fertilized at the optimum time of insemination with subsequent decrease in embryonic development [38]. Moreover, chronic oophoritis could resulted in changes in ovarian macrophage-like cytokines (tumor necroses factor 2α and interleukin-1) concentration which interfere with ovarian dynamics and conceivably alter the *In vitro* embryonic development [39].

In conclusion, this work debated the infection of camels with BVDV in Egypt. BVDV infection has great potential effects on ovarian dynamics, structures, IVF and embryos development in she camels. It is appeal with development of IVF and embryo production system in camels, all materials of animal origin must be screened against BVDV contamination. Further studies on other reproductive problems associated with BVDV infection of camels should be interested.

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