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Diagnosis of Acute Infection with Bovine Viral Diarrhea Virus in Dairy Cattle by Using Indirect ELISA and Immunohistochemistry Technique on Skin Biopsy Samples

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Abstract: Diagnosis of Bovine Viral Diarrhea Virus infection (BVDV) is an important step in control and eradication programs in cattle. Acute infection can result in deliver a persistent infected (PI) calf, secondary infection to BVD-linked immunosuppression or reproductive losses. The present work was carried out on a total number of 80 dairy Frisian cattle. Serum samples were examined for BVDV antibodies by using indirect Enzyme Linked Immunosorbent assay (ELISA). Ear notch tissue samples were taken for immunohistochemistry (IHC) by using strept avidin-biotin peroxidase complex method. A total number of 12 ear notch skin biopsy with high titre of BVDV IgG gave positive IHC results. Immunopositive reaction was observed as golden brown intra-cytoplasmic granules which were distinctive in cells of stratum germinativum and stratum spinosum of epidermis as well as hair follicles. Placenta of early aborted cases showed multiple areas of extensive coaggulative necrosis, inflammatory reaction and numerous clumps of bacterial colonies of mixed types. It could be concluded that indirect ELISA assay and IHC techniques on ear notch skin biopsy are efficient and reliable methods for detection of acute form of BVDV infection in cattle.

Key words: BVDV · Immunohistochemistry · ELISA · Pathology · Placenta

INTRODUCTION

Bovine Viral Diarrhoea Virus (BVDV) is a single stranded RNA virus and is member of Pesti virus genus of viral family flaviviridae [1, 2]. BVDV is an important viral disease in cattle causing economic losses in the herd [3] whereas it leads to early embryonic loss, abortion, still birth, congenital defects and birth of immunotolerant persistently infected (PI) calves [4]. BVDV caused immunosuppression, reproductive, digestive and respiratory disorders in cattle. Clinical conditions ranged from subclinical to severe haemorrhagic picture [5].

Currently, several methods are available for diagnosis of BVDV infection as cell culture isolation, antigen ELISA, RT-PCR and immunohistochemistry (IHC) technique. Since BVDV is a pantropic virus with a prominent epitheliotropism, enzyme labeled methods to detect BVDV antigens in tissue section by means of indirect immunohistochemistry (IHC) have been developed [6]. IHC on formalin fixed ear notch tissue samples has been shown to be useful method for identifying BVDV infected cattle. [7-11]. It could be distinguished between acute BVDV infected cattle and PI cases by indirect ELISA where the acutely infected cases become seropositive within 2-3 weeks post infection [12]. The aim of this study is diagnosis of acute BVDV infection in dairy cattle by using indirect ELISA assay and detection of virus antigen in ear notch skin biopsy by using IHC technique.

MATERIAL AND METHODS

Animals and History: The present study was carried out on total number 80 cows from a private dairy farm of Friesian cattle located at Giza province Egypt during the year 2014. These animals suffered from different reproductive disorders as; individual early abortion,

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infertility, reduction in milk and mastitis. These dairy cows proved to be negative sero-reactors for brucellosis using different serological tests.

Sampling

Blood Samples: Blood samples were collected from all examined animals by puncture of the jugular vein into sterile vacutainer tube for each sample. The obtained sera were examined for BVDV antibodies by using indirect ELISA assay.

Tissue Samples: Ear notch Skin biopsy samples sizing 2.0 cm were taken from the dorsal margin of pinna of examined cows using ear notcher for IHC carrying out. The ear notcher was disinfected by ethanol 70% among each cow [11]. Placenta samples were taken from three individual early aborted cases for histopathology.

Indirect ELISA

Concentration and Purification of Viral Strain: Reference viral strain was obtained from National Animal Diseases Laboratory (NADL) at titer $10^{5.4}$ Tissue Culture Infectious Dose ($TCID_{50}$) on MDBK (Madin-Darby bovine kidney). Cells were collected by cooling centrifugation at 8000 xg/30 min [13, 14].

Quantitation of Antigen Concentration: Total antigen concentration was estimated using total protein liquid color reagent (Stanbio laboratory, Boerue, Tx, USA) as in manufacturer's leaflet. The equation of antigen concentration formula was;

$$\frac{\text{Optical density (OP) of sample X Concentration of the standard (g/dl).}}{\text{OD of the Standard}}$$

Indirect Enzyme Linked Immunosorbant Assay (ELISA): Indirect ELISA carried out as described by Crowther [15] and according to checkerboard results. OD was read at 492 nm wavelength. Positive and negative controls were included in each plate as duplicated.

Cutoff Endpoint: OD summation of positive and negative controls was divided on number 4. Mean of OD > cutoff endpoint were considered positive. While, mean of OD = cutoff endpoint were considered negative.

Histopathological Examination: The samples were fixed in 10% neutral buffered formalin (NBF) for 24 hours, routinely processed, embedded in paraffin wax and sectioned at 5 μm [16].

Immunohistochemistry: A total number of 12 ear notch tissue samples were collected from the examined animals that showing high titre of anti BVDV antibody (1/512 and 1/1024) according to the indirect ELISA results. The specimens were fixed in 10% NBF for 24 hr. then to alcohol 70%. Tissue specimens were processed; paraffin embedded, sectioned at 5 mm and mounted on positively charged slides. Streptavidin/ biotin peroxidase kit imported from Scy Tek Lab. USA was used. IHC kit was species specified as anti-mouse, anti-rabbit. Antigen retrieval was occurred using Proteinase K (0.1%) [17]. Slides were incubated with mouse monoclonal anti P8o IgG (anti-nonstructural protein of BVD) as primary antibody imported from herdcheck IDEXX LAB. UK. Di Amino Benzedene (DAB) chromogen as a color indicator was applied.

RESULTS

Indirect Elisa Results: As shown in Table (1) a total number 80 serum samples were positive antibody titre (more than 1/256) in 38 cases (47.5%) and strongly positive (1/512 and more) in 12 cases (15%); from which ear notch skin biopsy was taken for IHC.

Table 1: Indirect ELISA results in BVDV infected dairy herd

Result	Titre	No (%)
Negative	Less than 1/ 128	30 (37.5%)
Positive	More than 1/256	38 (47.5%)
High Positive	1/ 512 and 1/1024	12 (15%)
Total	----------	80 (100%)

Immunohistochemistry and Histopathological Findings: A total number 12 ear notch skin biopsy samples with high titer indirect ELISA gave positive IHC. There was severely diffused golden brown immunostained BVDV antigen in most cells of stratum germinativum and stratum spinosum (Fig. 1, 2). More over; the high intensity of BVDV antigen was observed in the hair follicle in the dermal layer of skin more than epidermal layer (Fig. 3, 4). On the other hand, Placental tissues were negative with IHC for presence of antigen.

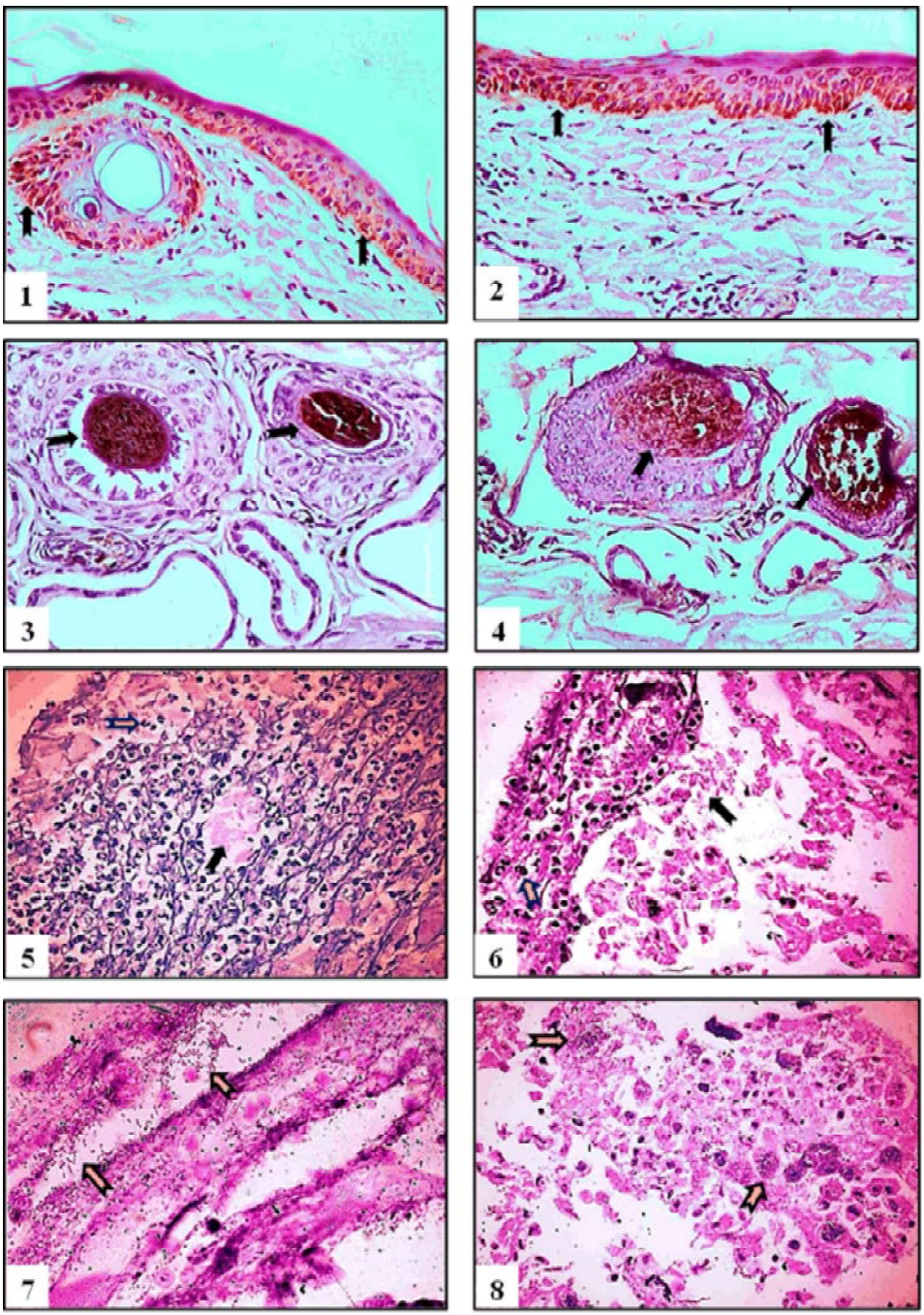

Fig. 1-4: (Fig. 1, 2) Cow; Ear notch skin biopsy. Fairly intense intra-cytoplasmic positive golden brown immunohistochemical staining of BVDV antigen was observed mainly in stratum germinativum and stratum spinosum of epidermis (arrows) H and E X 200. (Fig. 3 and 4) Cow; Ear notch skin biopsy. Strongly intense BVDV antigen localization in the hair matrix and in the lining epithelium of hair follicles (arrows). ABC technique, Di amino Benzedene chromogen (DAB) and hematoxylin counterstain X 200.

Fig. 5-8: (Fig. 5, 6) Placenta; cow. Myxomatus degeneration in the maternal crypts was pronounced (black arrow). Severe diffuse neutrophilic infiltration in interstitial tissue of chorioallantoic villi (yellow arrow), H and E X 200. (Fig. 7, 8) Placenta; cow. Showed either Diffuse scattered or multifocal clumps of bacterial colonies were observed in between necrosed chorioallantoic villi (yellow arrow) H and E X 100.

The histopathological examination of placenta showed necrobiotic changes of the epithelium lining of chorioalantoic villi. Multiple areas of extensive coaggulative necrosis associated with calcifications were seen. Myxomatus degeneration associated with extensive inflammatory reaction characterized by presence of intensive aggregations of neutrophils and some macrophages in the maternal crypts as well as in interstitial tissue of chorioallantoic villi (Fig.5 and 6). Numerous clumps of bacterial colonies of mixed types were seen (Fig.7 and 8).

DISCUSSION

The Acute form of BVDV infections was ranged from subclinical to fatal form for the infected animal, that according to the virulence of virus, immune status, reproductive status and other pathogens presence in the infected animals [18]. The way of transmission of infection in the absence of PI animals are not clearly understood but the contact with acutely infected animals could be explained the infection transmission [18].

Indirect ELISA considered as a reliable tool for screening of herds infected with BVDV especially when detected the anti P80 (non-structural protein antibody) [19]. Temporary viraemia in non-pregnant non immune cattle acutely infected with BVDV was occurred 3 days post infection and the immune response was developed 2 weeks later [20]. Indirect ELISA resulting in a high specificity and sensitivity 98% and 99% in diagnosis of BVDV infection as compared to serum neutralization test (SNT) [20, 21].

In the present study Indirect ELISA exhibited positive results in 47.5% and more over highly positive titre (1/512 and 1/1024) in 15% from the total number 80 examined cases. These results indicated that the high prevalence of antibody positive results reflect that the herd is in continuous state of infection due to presence of PI animals which act as viral reservoirs and subsequently the high seroprevalence of positive cases suggesting insufficiency of BVDV vaccine protection [12]. In this respect, it has been reported that antibody titre remains high for10 to 12 weeks post BVDV infection by using Indirect ELISA assay [12]. On the other hand, the cases of negative results of indirect ELISA assay in the presence work could be attributed to persistent infection with BVDV.

In the present study, BVDV antigen was detected intra-cytoplasmic in the basal cell layer of epidermis, in hair matrix and follicular cells of ear notch skin biopsy. IHC staining for BVDV antigen in formalin-fixed, paraffin embedded skin is considered as effective, reliable and screening method for diagnosis of infected cattle [7]. Ear notch skin biopsies have been reported as the sample of choice for testing young animals where the maternal antibodies do not interfere with detection of virus in skin biopsies [8, 22]. It has been found that 40% of acutely infected calves with BVDV had positive IHC staining in skin [8]. The variation in the intensity of BVDV antigen between the epidermis and hair follicular epithelium could be explained as; hair follicles are the predilection site for BVDV in infected cattle. Hair follicles that were arrested in telogen phase exhibited strong positive reaction for BVDV antigen. This is likely due to viral replication in hair matrix cells resulting in telogenization of hair follicles [7] In contrast; hair follicles that were positive for BVDV and in anagen phase may represent initial replication of the virus before the hair follicle entered telogen phase [23].

In the present work, the histopathological picture of placenta was characterized by multiple areas of extensive coaggulative necrosis, inflammatory reaction and numerous clumps of bacterial colonies of mixed types. However, Placental tissues were negative immunohistochemistry staining. In this respect, infection of a BVDV leads to a transient viraemia of 10–14 days duration [24]. This can be associated with short-term leukopenia [25], lymphopenia [18] which in turn, can allow other secondary infectious agents to become established or allow the recrudescence of existing infections. Immunosuppression is accompanied with direct effects of BVDV on circulating T and B lymphocytes [12] which decrease the capacity of the immune system to respond to other infectious agents.

CONCLUSIONS

It could be concluded that indirect ELISA assay and IHC techniques on ear notch skin biopsy are efficient and reliable methods for detection of acute form of BVDV infection in cattle.

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