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Distribution of bovine viral diarrhoea virus (BVDV) in the genital system tissues of cattle

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ABSTRACT

In this study, the cellular localisation and distribution of BVD viral antigens in genital system tissues were investigated in 65 non-pregnant dairy cows and in 65 bulls. For this study genital system tissue samples, taken from slaughtered bulls with no pathological lesions and from females having reproductive problems, were marked using the immunoperoxidase method on their paraffin sections. BVD viral antigens were determined in 15 of the 65 non-pregnant dairy cows (consistent with cell culture results) using the indirect immunoperoxidase method. BVD viral antigens were present in macrophage-like cells in the stroma of the ovaries and uterus. No BVD viral antigens were observed in the samples of testicles, epididymis, vesicula seminalis, or prostate in the male animals. In all animals, including BVDV-positive ones, no pathologic lesions were observed, except periodically non-specific subepithelial or stromal mononuclear cell infiltrations. Most of these cells were seen to be formed by lymphocytes and macrophages.

Key words: bovine viral diarrhea virus, immunohistochemistry, ovarium, uterus, testicle, epididymis

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Introduction

Bovine viral diarrhoea virus (BVDV) is a major cattle pathogen with a global distribution (BAKER, 1987; NETTLETON and ENTRICAN, 1995; WENGLER, 1991) and is responsible for a spectrum of symptoms and clinical disease, including reproductive failure. The immunosuppressive properties of BVDV may potentiate the pathogenic consequences of other microorganisms in complex disease of the respiratory and gastrointestinal tracts (BARKER et al., 1993; BROWNLIE, 1990).

Two pathogenic biotypes of BVDV, cytopathic (cp) and non cytopathic (ncp), have been described based on the presence or absence of visible cytopathic effect in vitro when monolayers are infected (BAKER, 1987; NETTLETON and ENTRICAN, 1995). Only ncp BVDV has been reliably shown to cross the placenta, invade the foetus and set up the persistent infection so crucial for successful virus spread (BROWNLIE, 1990; WENGLER, 1991). Recently, it has been established that viruses making up the Pestivirus species BVDV belong to two different genotypes known as BVDV-1 and BVDV-2. Like the BVDV-1 viruses, BVDV-2 viruses may exist as one of two biotypes (SCHERER et al., 2001).

The pathogenesis of BVDV infections shows characteristic features not seen in other diseases. Singly or in combination the two biotypes of bovine viral diarrhoea virus induce clinical disease of multiple organ systems (BARKER et al., 1993). Pre- and post-natal infections are associated with a variety of disease syndromes which include diarrhoea, abortion, congenital defects, immunosuppression, mucosal disease (MD) and disruption of reproductive function, the latter arguably being of most importance. The lesions best characterized are those associated with the rare and highly fatal mucosal disease and chronic BVD (BAKER, 1987; NETTLETON and ENTRICAN, 1995). However, acute infection with a highly virulent BVDV-2 was associated with prolonged high fever and sudden death (peracute BVD), and enteric disease with lesions similar to those of mucosal disease (BOLIN and RIDPATH, 1992; RIDPATH et al., 2000; STOFFREGEN et al., 2000). Also, thrombocytopenia and the resulting hemorrhagic syndrome have been most often associated with infection of cattle with BVDV-2 (CORAPI et al., 1990; MARSHALL et al., 1996).

Similar lesion are seen in the early stage of naturally occurring MD and acute BVD infections, and are usually accompanied by inflammation of the lamina propria of oral and nasal mucosa, because initial infection and replication occur in the oronasal mucosa and oropharyngeal lymphoid tissues. Systemic spread of extracellular virus and virus within leukocytes occur in lymphatic and blood vessels (BARKER et al., 1993; RIDPATH et al., 2000). BVDV infected cells in a different ratio, because BVD viral antigens were distributed discriminately to different organs and lymphoid tissues (LIEBLER-TENORIO et al., 1997).

Many studies which were to use immunohistochemical techniques have addressed the distribution of virus in various organs from persistently infected (PI) or acutely infected animals (FERNANDEZ et al., 1989; THUR et al., 1996; HOUE, 1999; WILHELMSEN et al., 1990; WILHELMSEN et al., 1991; HEWICKER et al., 1990; MARSHALL et al., 1996; BASZLER et al., 1995; FREDRIKSEN et al., 1999). However, the tissue and cellular distribution of BVDV antigen in the genital system from PI or acutely infected animals has received less attention. Recently, ovaries have been shown to be one of the possible areas of BVD virus replication, and this could lead to abnormal ovum development (FRAY et al., 1998; GROOMS et al., 1996).

In this study, distribution and cellular localisation of BVD viral antigens was investigated in paraffin-embedded genital system tissue samples taken from male and female animals. The animals had previously been used in the study of AK et al. (2002) for determining the prevalence of BVDV infection.

Materials and methods

Animals. Samples of 130 cattle (65 samples from bulls, 65 samples from dairy cows) were collected from the slaughterhouse. The age of the bulls ranged from 10 to 18 months. These animals were generally food animals with no apparent problem. Tissues obtained from bulls were testes, epididymis, prostate, and vesicula seminalis. The ages of the cows ranged from 4 to 6 years. In general these animals were brought to the slaughterhouse due to infertility problems. Samples of ovaries and uterus were taken from cows.

The blood samples of these animals were used in a previous study; BVDV were found in 4 of 65 bulls and 15 of 65 dairy cows by indirect immunoperoxidase test in cell cultures (AK et al., 2002)

Immunoperoxidase staining kit. The peroxidase test was performed using a commercial kit (IPEX-BVD kit, Central Veterinary Laboratory, New Haw, Addlestone, Surrey KTI53NB, United Kingdom). The kit contained 0.01 M PBS (pH 7.6), dilution fluid, monoclonal antibodies mix (MABmix; ASC WB112, ASC WB166, ASC WB103, ASC WB214), rabbit anti mouse Ig/HRPO conjugate, substrate (diaminobenzidine tetrahydrochloride) and O_2 donor (sodium perborate tetrahydrate).

Histopathology. The tissue samples from genital systems were fixed in neutral buffered 10% formalin for 18 hours, processed in a grated concentration of ethanol and embedded in paraffin. Sections were cut from uterus and both left and right ovaries and then stained with hematoxylin and eosin (HE). Ziehl-Neelsen staining was applied to those considered necessary (LUNA, 1968).

Indirect immunoperoxidase test (IIP) formalin-fixed tissues. Tissues were processed for IIP as previously described (FERNANDEZ et al., 1989). Paraffin-embedded tissue blocks were sectioned at 3-4 ìm, mounted on poly-L-lysine treated microscope slides, and immunoperoxidase staining of sections was performed using IPEX- BVD KIT. Prior to incubation with Mabmix, sections were incubated with 3% H_2O_2 to quench endogenous peroxidase activity, enzymatically treated with 0.1% trypsin (Sigma) and incubated with 1% BSA (Sigma). After incubation with rabbit anti mouse Ig/HRPO conjugate (CVL) immunostaining was visualised by diaminobenzidine tetrahydrochloride substrate (CVL). Slides were stained with Mayer's hematoxyline and coverslipped with an aqueous mounting medium (JACSON and BLYTHE, 1995). Excluding Mabmix the serial sections of the immuno-stained sections were also stained for controlling non-specific binding of secondary antibody. The coverslips were washed and counterstained with Harris hematoxyline.

Results

Histopathological findings. In the general histopathological examination carried out with the HE staining of paraffin blocks of the ovarium

Table 1. Specification of myomorphus mammals examined by renoculture and microscopic agglutination acording to the trapping area with corresponding results

and uterus belonging to cows brought to the slaughterhouse, endometritis caseosa lesions were observed in the uterus of 3 animals. In 2 of these animals granulomatose inflammation characterised with giant cell formation was determined. In the investigation with regard to acido-resistance bacteria

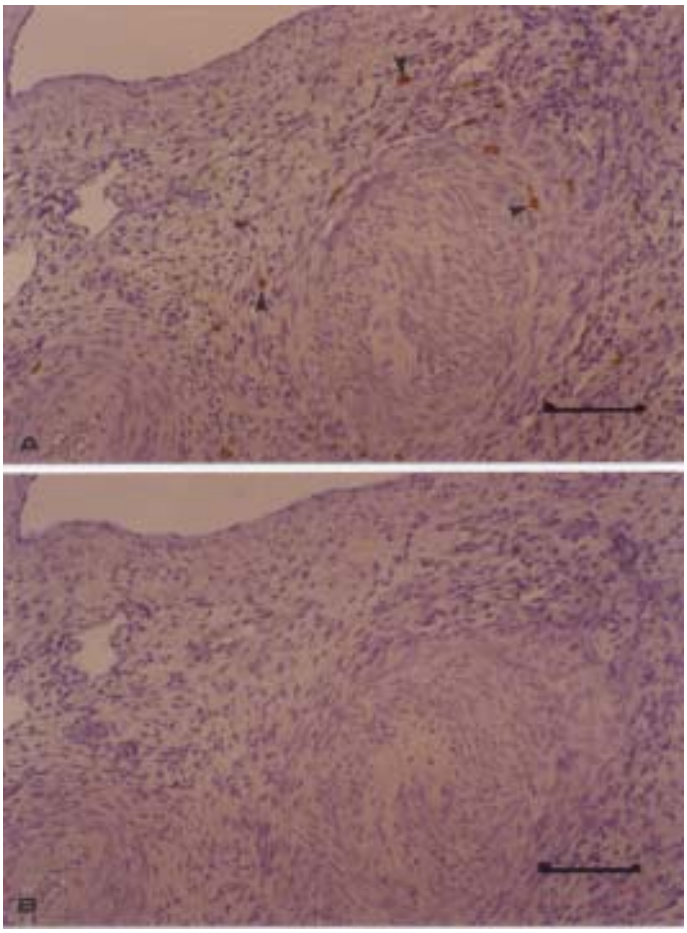

Fig. 1. Ovarian sections, immunohistochemical labelling. A) BVDV antigen-positive cells are located around the arterioles (arrow heads). 230x; bar 100 μm
B) Control section. Labelling is not present 230x; bar 100 μm.

of these tissue blocks with granulomatose endometritis, these bacteria were determined in only one sample. In male animals, orchitis was determined in one testis and testis atrophy in two testis tissues. Apart from these findings, no lesion was found related to a specific pathogen in the samples obtained from cows and male animals.

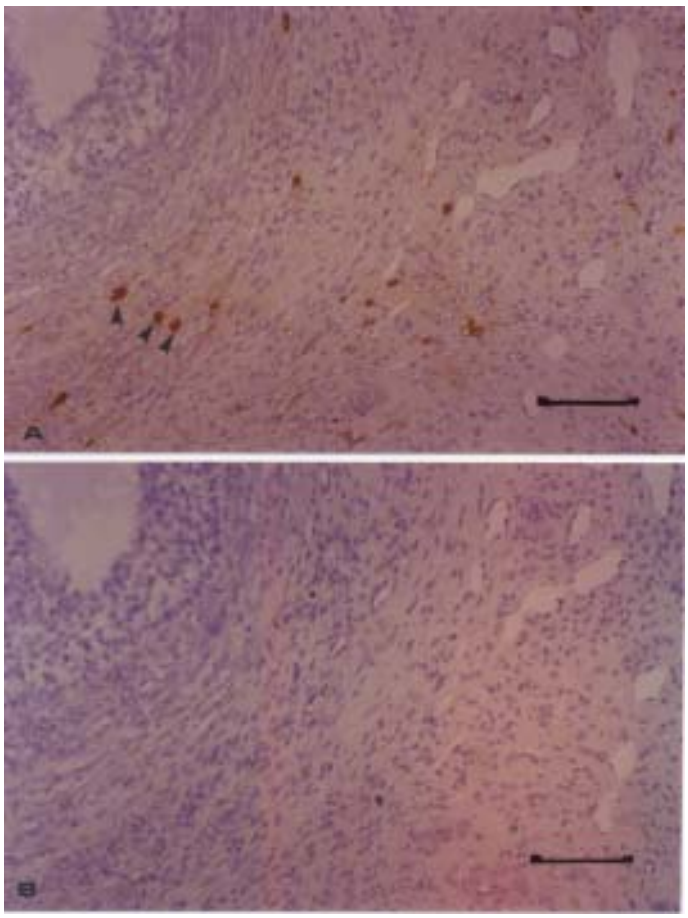

Fig. 2. Ovarian section, immunohistochemical labelling. A) Location of BVDV-positive cells in medullar stroma (arrow heads). 230x; bar 100 μm. B) Control section. Labelling is not present 230x; bar 100 μm.

Marking of tissue blocks with immunoperoxidase. In the marking of tissue blocks with immunoperoxidase, positivity with regard to BVDV was determined in 15 female animals. The distribution of this positivity was in the ovarium and uterus in five, in the ovarium in eight, and in the uterus

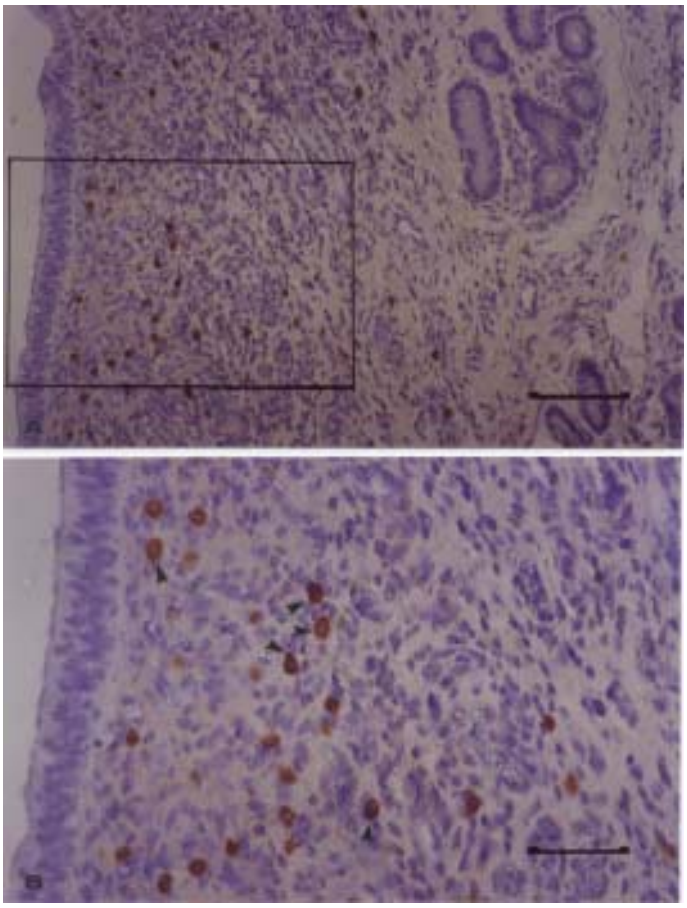

Fig. 3. Uterus sections, immunohistochemical labelling. A) BVDV antigen-positive cells are located in superficial L. propria (arrows). Labelling is not present in epithelial cells of the lining epithelium and the glands. 230x; bar 100 μm. B) Higher magnification of A 460x; bar 50 μm.

in only two female animals. The positive markings in the ovarium were mostly seen in the granular type in mononuclear cells immediately surrounding the arterioles and veins in the medullary stroma (Fig. 1) and in the remaining connective tissue cells (Fig. 2). It was observed that the positive markings in the uterus tissue were particularly in the cytoplasma of the macrophage-like cells in the stroma of the superficial lamina propria mucosa without a clear histopathological lesion (Fig. 3).

In the ovaria, in which BVDV positive markings were determined, no obvious lesion was observed except a relatively low quantity of tertiary and graafian follicles and slight mononuclear cell infiltrations in the superficial lamina propria mucosa in the uterus samples. Positive markings were not determined in any of the male animal tissue samples. Labelling was not observed in the Mabmix excluded sections.

Discussion

BVDV pathology studies predominantly focus on the establishment of persistent infections and the development of mucosal disease or chronic BVD. Calves persistently infected with BVDV contain viral antigen within cells of skin, neurones of the cerebral cortex, lymphoid organs, and cells of mononuclear phagocyte system of non-lymphoid organ, such as liver, kidney, lung, and the alimentary tract (THUR et al., 1996; HEWICKER et al., 1990; SHIN and ACLAND, 2001; WILHELMSEN et al., 1991). In cases of MD, BVDV antigen was also detected in many different cell types in a variety of organ tissues and was not always associated with tissue alteration (FERNANDEZ et al., 1989; LIEBLER-TENORIO et al., 1997).

WILHELMSEN et al. (1991) examined the tissue lesions and distribution of BVDV in various systems from cattle with naturally acquired MD, chronic BVD, and persistent infection. The gross and microscopic lesions were different in cattle with either form of clinical disease. However, localisation of BVD viral antigen was similar in both clinical forms. Intracytoplasmic BVD viral antigen was detected both in mononuclear cells and parenchymal cells. In the present study, gross lesions in the examined organs and specific pathological lesions in the tissue sections from BVDV antigen positive animals were not detected. The only significant abnormality was in occasional subepithelial and stromal cellular infiltration in HE- stained paraffin

sections of uterus, ovaries, prostate and vesicula seminalis. This infiltration is composed of predominantly mononuclear cells, which are lymphocytes and macrophages.

Relatively few studies have focused on the localisation of virus in the genital organs from PI animals. It is known that in persistent infection the virus has a broad tissue tropism and infects many cell types, including mononuclear cells within the wall of blood vessels, granulosa cell, luteal cells, and oocytes in ovary, gland epithelium in uterus, placenta, and pituitary gonadotrophs. BVD viral antigen was seen in mononuclear cells, with or without causing microscopic lesions (FERNANDEZ et al., 1989; FRAY et al., 1998; LIEBLER-TENORIO et al., 1997). In the ovaries, BVDV antigen was detected in the follicular epithelia in the tertiary follicles. Antigens were not detected in ova in primordial and secondary follicles (SHIN and ACLAND, 2001). However, other studies (BASZLER et al., 1995; FREDRIKSEN et al., 1999) reported that the pattern of BVDV replication seen within the oocyte population is consistent with the sporadic localisation of BVDV within the ovary, placenta, alimentary tract, and leukocytes. In addition, FREDRIKSEN et al. (1999) and SCHERER et al. (2001) reported that pregnancy appeared to favour virus replication in the uterus, as the sections from pregnant heifers showed much stronger staining in the epithelium of endometrium and placentomes and a higher proportion of viral antigen-positive cells than sections from non-pregnant PI heifers. GROOMS et al. (1996) detected BVDV antigens in the luteal cells and macrophage-like cells in the ovaries of PI animals, at the same time as a decrease in number of follicles and corpus hemorrhagicum/luteum/albicans was observed.

The present study showed that BVDV-specific antigen was consistently found in macrophage-like cells lining in the stroma and around the theca externa of ovaries, and in the subepithelial stroma and around the blood vessels of the mucosa of uterus, and were not found in parenchymal cells of organs. In addition, there was no obvious lesion other than the relatively low number of tertiary and graafian follicles in ovaria with BVDV-positive marking and slight mononuclear cell infiltrations in the superficial lamina propria mucosa in uterus samples. This contrast may be created by the possible presence of a different biotype of the virus and different clinical form in our cases, as well as postnatal acute infection.

Primary postnatal acute infections of BVDV are common in cattle population, but the distribution of virus in cells of the host during the course of a primary acute infection is not fully understood (MARSHALL et al., 1996; BRUSCHKE et al., 1998; LIEBLER-TENORIO et al., 1997). There are few previous immunohistochemical studies related to acute BVDV infection in cattle regarding localisation of the virus antigen (STOFFREGEN et al., 2000). WILHELMSEN et al. (1990) reported that the two viral biotypes used in their study induced similar gross and microscopic lesions, oedema to necrosis and inflammation in the lymphoid, enteric, and respiratory systems. Intracytoplasmic BVD viral antigen-bearing cells were found randomly in mononuclear cells within the gut- and bronchus-associated lymphoid tissue, and in the cortex, paracortex, and medulla of mesenteric lymph nodes. MARSHALL et al. (1996) reported that viral antigen was also detected in the cytoplasm of mononuclear cells in the bronchus-associated lymphoid tissues and in splenic red pulp, in the theca externa of ovarium, and the squamous epithelium cells of rumen and tonsilar crypts. BVD viral antigen was seen in mononuclear cells, causing various microscopic lesions as well as oedema to necrosis in the lymphoid tissues and in the alimentary tract.

By comparison, the present study showed that gross lesions in the examined organs and specific pathological lesions in the tissue sections from BVDV antigen-positive animals were not detected. However, BVDV-specific antigen was consistently found in macrophage-like cells lining the stroma and around the theca externa of ovarium, in the superficial stroma and around the blood vessels of the mucosa of uterus. This contrast to the results of MARSHALL et al. (1996) and WILHELMSEN et al. (1990) may be created by the possible presence of different biotype or virulence of the virus in our cases, and may also form the basis for secondary diseases involving multiple pathogens in other cases (BARKER et al., 1993; MARSHALL et al., 1996). Since the materials for this study were collected from a slaughterhouse they were from naturally infected animals. Due to the above discrepancies with the literature, that a full complement of tissues was not examined and that double labelling of antigen-bearing cells was not carried out, we could not precisely determine the clinical form of BVDV infection.

In addition the importance of BVDV infection in males is the shedding of BVDV by semen of both acutely infected and PI bulls. The titre of virus

in semen, as well as in tissue and blood after acute and persistent BVDV-infection, has been shown to be different as gross and microscopic lesions (KIRKLAND et al., 1997; KOMMISRUD et al., 1996). BVDV can contaminate the female genital system both by artificial insemination (AI) and coitus (MEYLING and MIKEL-JENSEN, 1988). Semen from bulls that are acutely infected with BVDV has also been found to contain the virus 2 to 14 days post-infection. Most acutely infected bulls which have no pathologic change have normal semen quality. BVDV antigens were identified in the glandular epithelial cells of the prostate gland, the seminal vesicles and ampullae from acutely infected and PI animals. However, antigen was isolated from testis and the epididymis of only one PI bull (KIRKLAND et al., 1991).

In the present study BVD viral antigens were not seen in the samples of testicles, epididymis, vesicula seminalis or prostate in the male animals (inconsistent with the cell culture results). Different percentage of positive labelling between immunocytochemistry and immunohistochemistry was reported in animals (BASZLER et al., 1995; KIRKLAND et al., 1991). Results of the present study are in accordance with these studies.

Our findings of determining antigen positivity in mononuclear-like cells except tissue parenchymal cells in the ovary, uterus or both tissue samples in female animals with antigen-positive leucocytes support that, with respect to the females examined, the animals were natural acutely infected animals, that the positivity present indicated random distribution with circulation of viruses related to leucocytes. At this point, the absence of positivity in male animal tissues with positive leucocytes suggests that this may have caused incorrect negative results in immunohistochemical examination in limited investigation sites due to the random distribution of the virus.

Also, virus isolation using immunocytochemistry on organ material in BVDV infections will cause positive results in both PI and acutely infected animals due to the material containing blood. Although persistent infection can be determined by double blood collection from live animals using immunocytochemistry, in slaughtered material immunohistochemistry is believed to be efficient in differentiating acute and persistent BVDV infections only if antigen-bearing cells are identified with double labelling.

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SAŽETAK

Istražena je lokalizacija i proširenost antigena virusa virusnog proljeva goveda u tkivima spolnog sustava 65 negravidnih mliječnih krava i 65 bikova. U tu svrhu rabljen je imunoperoksidazni test na histološkim rezovima tkiva. Tom metodom pretraženi su uzorci tkiva spolnih organa uzeti od zdravih zaklanih bikova te krava s reprodukcijskim poremećajima. Virusni antigeni dokazani su u 15 od 65 krava (sukladno s uzgojem virusa na staničnoj kulturi). Antigeni su bili prisutni u makrofagima sličnim stanicama unutar strome jajnika i maternice. Virusni antigeni nisu dokazani u uzorcima tkiva testisa, epididimisa, sjemene vrećice i prostate bikova. U pretraženih životinja, uključujući i one pozitivne na virus, nisu zabilježene patološke promjene, osim povremenih nespecifičnih subepitelijalnih ili stromalnih mononuklearnih infiltracija limfocitima i makrofagima.

Ključne riječi: virusni proljev goveda, imunohistokemija, jajnik, maternica, testis, epididimis