Distribution of viral antigen and development of lesions after experimental infection with highly virulent bovine viral diarrhea virus type 2 in calves

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Objective—To correlate tissue distribution with development of lesions after experimental infection with a virulent strain of noncytopathic bovine viral diarrhea virus (BVDV) type 2 in calves.

Animals—Ten 14-day-old and two 2-month-old colostrum-deprived calves.

Procedure—Calves were intranasally inoculated with BVDV type-2 strain 1373 from an outbreak of clinically severe bovine viral diarrhea (BVD). Two 14-day-old calves served as noninfected controls. Two calves each were euthanatized on postinoculation days 3, 6, and 12, and 1 each on days 8, 9, 13, and 14. Tissues were collected for immunohistologic and histologic examination.

Results—Inoculated calves developed nonspecific clinical signs characterized by high fever and decreased numbers of leukocytes and thrombocytes. Viral antigen was detected focally in lymphoid tissues on day 3. On days 6, 8, 9, 12, and 14, viral antigen became increasingly widespread throughout organs and tissues. Viral antigen in lymphoid tissues was associated with severe depletion of all compartments. Lesions in other tissues were not well correlated with distribution of viral antigen. Depletion of lymphoid tissues was observed in a calf on day 13, but viral antigen had been cleared from most tissues and was detected in vascular walls only.

Conclusions and Clinical Relevance—Infection with a virulent BVDV strain resulted in widespread dissemination of viral antigen in host tissues. Severe lymphoid depletion developed in lymphoid tissues, whereas viral antigen was generally not associated with lesions in other tissues. Findings suggest that development of lesions in acute BVD is not solely a function of viral replication and is also attributable to host reaction to infection. (Am J Vet Res 2002;63:1575–1584)
of undefined species investigated the distribution of viral antigen in the early phase of infection without relating its presence to tissue lesions. The other study evaluated lesions and viral antigen in calves between day 6 and 10 after inoculation with BVDV 2 without attributing them to duration of infection. The objective of the study reported here was to correlate spread of a virulent noncytopathic BVDV 2 with development of clinical signs and tissue lesions at consecutive times after experimental infection in calves.

**Materials and Methods**

**Calves**—Ten 14-day-old and two 2-month-old colostrum-deprived healthy calves of different breeds were used. Calves were procured from area dairies at birth and raised in individual pens. During the first weeks, they were fed a milk replacer twice per day, then milled corn and oats. They had access to water and hay ad libitum. Animal studies were done in compliance with the Animal Welfare Act, US Public Health Service Policy on the Humane Care and Use of Laboratory Animals, NRS Guide for the Care and Use of Laboratory Animals, and Guide for the Care and Use of Agricultural Research and Teaching (FASS 1999). All calves tested negative for BVDV, as determined by use of virus isolation followed by polymerase chain reaction (PCR) assay, and did not have antibodies against BVDV as determined by use of serum neutralization with BVDV type-1 strain NY-1 and BVDV type-2 strain 1373.

**Inoculum**—Noncytopathic BVDV type-2 strain 1373 used for inoculation was an isolate from the widespread outbreak of clinically severe acute BVDV infection in Ontario, Canada, in 1993. It induced severe clinical disease in preceding experiments.

**Experimental procedure**—Calves were kept in confinement in individual pens and fed as described. Ten calves received 5 mL of 10^6 tissue culture infective doses (TCIDs) of BVDV 2 strain 1373 administered intranasally. Clinical condition was monitored twice and rectal temperature once per day. Blood samples were collected into tubes containing anticoagulant at the time of inoculation and then every other day. In these samples, the numbers of leukocytes, lymphocytes, and thrombocytes were determined, and theuffy coat was examined for BVDV by use of virus isolation followed by PCR assay. Two calves each were euthanatized on postinoculation days (PIDs) 3, 6, and 12 and 1 each on PIDs 8, 9, 13, and 14. Two calves were not inoculated and served as controls.

**Histology and immunohistology**—Tissue samples were collected from 38 locations including lymphoid organs (tonsil, mandibular lymph node, retropharyngeal lymph node, subcapsular lymph node, mesenteric lymph node, spleen, and thymus), digestive tract (oral mucosa, esophagus, rumen, abomasum, duodenum, jejenum, colon, Peyer’s patches in jejunum and ileum, lymphoid glandular complexes in the colon and rectum, parotid salivary gland, pancreas, and liver), respiratory tract (nasal orifice, turbinates, trachea, and lung), endocrine organs (thyroid gland, pituitary gland, pancreas, and adrenal glands), urogenital organs (kidney, testis), CNS (cerebrum, hippocampus, and cerebellum), heart, skin, interdigital skin, and bone marrow. Two samples were collected from each site. One sample was fixed in neutral-buffered 3% formalin, embedded, and sectioned; the other sample was snap-frozen in isopentane at −70°C for immunohistologic examination. The embedded sections were stained with H&E. Viral antigen was detected in cryostat sections by use of the indirect immunoperoxidase method as described. Monoclonal antibody B252 that recognizes an epitope on E2 and monoclonal antibody BVD/C16 that recognizes an epitope on NS2-3 were used as primary antibodies. Both monoclonal antibodies were in cell culture supernatants that were diluted 1:2 in PBS solution (pH, 7.1) that contained 1% Tween-20.

**Results**

**Clinical signs**—Three of the 10 inoculated calves had increased rectal temperature that was > 40°C on PID 2; in 4 calves, a temperature rise was recorded on PID 7 or 8. One calf had rectal temperature within reference range during the entire observation time of 14 days. Although rectal temperature varied widely, there was a clear difference between control and infected calves (Fig 1). Calves became progressively listless and lethargic after inoculation. Diarrhea containing mucus and blood was seen in 5 calves beginning between PIDs 5 and 10. One calf became recumbent on PID 8 and was therefore euthanatized and necropsied 1 day earlier than planned.

A decrease in WBCs was noted as early as PID 3 in 8 of the 10 inoculated calves. With the exception of 1 calf that became moribund, lymphocyte numbers fluctuated widely without a distinct pattern in the 2-week-old calves (Fig 2). A continuous decrease in lymphocytes to < 60% of baseline values was detected in one 14-day-old calf and in the two 2-month-old calves.
between PIDs 5 and 9. This was followed by a further
decline to < 88% of baseline values.
Thrombocytes decreased to < 60% of baseline val-
ues in 3 of the 2-week-old calves and the two 2-month-
old calves between PIDs 7 and 10 (Fig 2). In 1 calf, the
number of thrombocytes had decreased to 29,000
cells/µL at PID 8, a 94% reduction. In all calves, BVDV
could be isolated from the buffy coat beginning at PID
2 or 3 and until necropsy was performed.

Macroscopic findings—Lesions detected at
necropsy, even in severely lethargic calves, were mild.
Four calves were dehydrated and had severely sunken

Table 1—Lymphoid and intestinal tissue distribution of viral antigen on various postinoculation days
(PID) after inoculation with bovine viral diarrhea virus (BVDV) 2 strain 1373 in calves

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*Only cells in the interstitium. *Only in the lymphoid tissue. *Only vascular walls or perivascular infiltrates. – = Viral antigen
not detected. + = Viral antigen detected. Results in each column represent findings in 1 calf.

flanks. Tissue lesions included petechial hemorrhages
and depletion of lymphoid tissues. Petechiae were seen
as early as PID 3 in lymph nodes. In the calves necrop-
sied on PIDs 6, 8, 9, 12, and 14, petechial hemorrhages
were commonly encountered. In the calf necropsied on
PID 13, only lymphoid depletion of the Peyer’s patch in
the ileum and of the thymus were noted. A circum-
scribed catarrhal bronchopneumonia was detected in 1
cranial lung lobe in 3 calves.

Distribution of viral antigen—Viral antigen was
detected in the lymphoid tissues of both calves necrop-
sied on PID 3 in scattered mononuclear cells through-
out lymphoid tissues and in lymphocytes of a few lymph-
oid follicles in the tonsils, lymph nodes, and
mucosa-associated lymphoid nodules in the small and
large intestine (Table 1; Fig 3). Some of the lymphoid
folicles that contained viral antigen were mildly depleted of lymphocytes. In the tonsils, viral antigen was also distributed multifocally in the epithelium. In the mucosa-associated lymphoid nodules, viral antigen was detected in 1 calf in the lymphoid follicles only, whereas a few epithelial cells in the mucosa overlying the lymphoid follicle also contained viral antigen in another calf. Viral antigen was detected in a multifocal distribution in cells with dendritic morphologic features in the thymic cortex. Numerous mononuclear cells contained viral antigen in the spleen of 1 calf. The bone marrow yielded negative results for viral antigen in both calves necropsied on PID 3.

On PID 6, the amount of viral antigen in the lymphoid tissues was greater than previously detected and was detected in cells in all compartments of the lymphoid organs, including most lymphoid follicles (Fig 4). In addition, viral antigen was detected in all parts of the digestive tract, including rumen and abomasum, where it was detected in epithelial cells and mononuclear cells in the lamina propria (Tables 1 and 2; Fig 5). Viral antigen was detected multifocally in epithelial cells and in subepithelial mononuclear cells of the oral mucosa, esophagus, turbinates, and trachea, and in cells in the interstitium of the lung. Numerous hematopoietic precursor cells and megakaryocytes in the bone marrow contained viral antigen (Fig 6). Viral antigen was detected in pancreatic islets of both calves and in the adrenal medulla of 1 calf.

On PIDs 8, 9, 12, and 14, viral antigen was com-

Table 2—Distribution of viral antigen in various tissues on various PIDs after inoculation with BVDV 2 strain 1373 in calves

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<th>Tissue</th>
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*Only vascular walls, perivascular infiltrates. †Only cells in the interstitium. See Table 1 for remainder of key.

Figure 5—Photomicrograph of a section of the jejunum of a calf 6 days after experimental inoculation with BVDV. Notice that BVDV antigen is distributed multifocally in crypt epithelium and in lymphocytes and macrophages in the lamina propria. Immunohistochemical stain; bar = 100 µm.
Viral antigen was commonly detectable in endocrine organs. Antigen was detected in a multifocal to diffuse distribution in the adrenal medulla and in a focal to multifocal distribution in the interstitium of the adrenal cortex of 3 calves. A few islets with antigen were seen in the pancreas of 1 calf, many were detected in 2 calves, and islets and acinar epithelium contained antigen in 2 calves. Viral antigen that was distributed multifocally to diffusely was detected in the neurohypophysis of 4 calves, and antigen distributed focally to multifocally was detected in the adenohypophysis of 3 calves. In the thyroid gland, viral antigen was contained predominantly in cells in the interstitium, and antigen was detected in the thyroid epithelium only in 1 calf.

In the kidneys and testes, variable numbers of cells in the interstitium contained viral antigen; in the kidneys, only a few tubular epithelial cells and glomeruli in 2 calves contained antigen. Viral antigen was evident in vascular walls and mononuclear cells, especially in the portal triads of the liver. In the heart, viral antigen was detected in vascular walls, mononuclear cells in the interstitium, and myocardial cells of 3 calves (Fig 10). Viral antigen was evident in epithelial cells of the interdigital skin in 5 calves. In skin collected from the upper portion of the hind limb, viral antigen was detected in mononuclear cells in the dermis of 3 calves and in epidermal cells of the skin in 2 calves.

In the cerebrum and hippocampus, viral antigen was detectable only in the vascular wall of 1 arteriole in the hippocampus of 1 calf. A few cells in the leptomeninges and in the Purkinje cell layer of the cerebellum contained viral antigen in 4 calves.

In the calf necropsied on PID 13, viral antigen was predominantly detected in vascular walls of various tissues such as lymph nodes and the submucosa and muscularis of the digestive tract, as well as the lungs, heart, and pancreas (Table 1 and 2). Viral antigen in

Figure 6—Photomicrograph of a section of bone marrow of a calf 6 days after experimental inoculation with BVDV. Notice that BVDV antigen is in several megakaryocytes (arrows) and many myeloid cells. Viral antigen is not evident in some megakaryocytes (arrowheads). Immunohistochemical stain; bar = 50 µm.
this calf was associated with severe vascular lesions. In addition, viral antigen was focally detected in the lymphoid tissue of the tonsils and in a few cells in the interstitium of the kidney.

Histologic findings—Lesions were predominantly seen in lymphoid tissues. In 1 of the calves necropsied on PID 3, there was mild lymphoid depletion of a few lymphoid follicles in the tonsils and the retropharyngeal lymph node. On PIDs 6 and 8, lymphoid follicles in all lymphoid tissues, including the Peyer’s patches, had lymphoid depletion that varied in severity. At the same time, there was lymphocytic depletion in the T-cell compartments of the tonsils, lymph nodes, and spleen. Moderate lymphoid depletion of the thymic cortex was evident in 1 calf on PID 6, and severe depletion was seen in 1 calf on PID 8. On PIDs 9, 12, 13, and 14, small lymphoid follicles or lymphoid follicles with moderately depleted centers were seen in most lymphoid tissues. Lymphoid follicles were severely depleted only in the jejunal Peyer’s patches of 3 calves and in the ileal Peyer’s patches of 5 calves. There was moderate to severe lymphocytic depletion in the T-cell compartments. An accumulation of apoptotic cells and cystic changes in lymphoid follicles were seen in the proximal portion of the colon of 3 calves. The thymic cortex was mildly depleted in 1 calf, moderately depleted in 1 calf, and severely and diffusely depleted in 3 calves. In 1 calf, the thymic medulla was also depleted. The bone marrow was moderately depleted in the calves necropsied on PIDs 8, 9, 12, and 13 and severely depleted in the calf necropsied on PID 14.

Beginning on PID 9, mucosal lesions characterized by elongated crypts with increased numbers of mitotic...
and apoptotic crypt epithelial cells were evident in all portions of the small and large intestine. The number of goblet cells was markedly reduced in the large intestine. On PIDs 12 and 14, distorted crypts with increased numbers of apoptotic epithelial cells and accumulation of cellular debris in the crypt lumina, focal destruction of crypts, moderate villus atrophy, and severe villus fusion in the small intestine were seen. An increased number of apoptotic cells was detected in the basal cell layer of the oral mucosa, esophagus, and rumen; in the crypts of the abomasum.
of 3 calves; and in the basal cell layer of the skin, inter- 
digital skin, nasal orifice, turbinates, and trachea of 1 
calf.

In 6 of the 12 calves, a mild multifocal nonpurulent 
interstitial nephritis was detected. In 3 calves, a 
catarrhal to fibrinopurulent bronchopneumonia was 
present. Perivascular lymphohistiocytic infiltrates and 
segmental fibrinoid necrosis were seen in small and 
medium-sized arteries of various lymph nodes, ileal 
Peyer’s patches, rumen, myocardium, and bone mar-
row on PID 13 (Fig 11).

Discussion

Inoculation of calves with noncytopathic BVDV 2 
strain 1373 resulted in clinical signs characteristic of 
acute BVDV infection, such as fever, diarrhea, and 
signs of depression. The severity of these clinical 
signs caused recumbency in 1 calf on PID 8, and the 
marked decrease in circulating lymphocytes and 
thrombocytes confirmed that the BVDV strain used for 
inoculation, which had been isolated from an outbreak 
in Canada, had retained its high virulence. As report-
ed on PID 6. This is later than in all other lympho-
dermal tissues, reported without viral antigen being 
detected without associated lesions. Tissue alterations, 
including fever, nonspecific clinical signs, and inconspic-
uous macroscopic lesions.

Examination of 2 calves in the early phase of in-
fec tion revealed that viral antigen was in the epithelium 
and the underlying lymphoid tissue of the tonsils as 
early as PID 3. This indicates that tonsillar crypt 
epithelium is most likely the site of entry of noncyto-
pathic virus after intranasal inoculation, which is simi-
lar to cytopathic BVDV during mucosal disease. 

From the tonsils, lympho-hematogenous spread to 
other lymphoid tissues occurs, as indicated by the mul-
tifocal distribution of viral antigen in various other 
lymphoid tissues. Gut-associated lymphoid tissues 
such as Peyer’s patches are also infected via the 
haftogenous route, because in 1 of the calves necrop-
sied on PID 3 only lymphoid tissue, but not epithel-
ium, contained viral antigen.

As reported after the inoculation of calves with a 
noncytopathic BVDV strain of unknown identity, a 
marked increase in the amount of viral antigen was 
found on PID 6 in the lymphoid tissues. Viral antigen 
was especially abundant within lymphoid follicles, but 
was not limited to lymphoid follicles as reported for 
cytopathic BVDV during mucosal disease; in the calves reported here, antigen was detected in all 
 compartments of the lymphoid tissues.

In the bone marrow, viral antigen was first detect-
ed on PID 6. This is later than in all other lympho-
 hematopoietic tissues. Reports about the infection of 
myeloid cells and megakaryocytes in the bone mar-
row vary depending on the viral strains used. After 
infection with BVDV 2 strain 1373, BVDV antigen was 
widespread in the bone marrow of all our calves from 
PID 6 onward, except in 1 calf in which the virus was 
not detected in most tissues on PID 13. This indicates 
that transient infections of the bone marrow may 
occur, as observed by others.

Viral antigen was not restricted to lympho-
 hematopoietic tissues and was also detected in the res-
piratory tract, digestive tract mucosa, pancreas, and 
adrenal gland on PID 6 and in most tissues after PID 8. 
This widespread distribution of viral antigen is consist-
ent with reports from other experimental infections 
that resulted in clinical disease. After infection with 
viral strains that induced mild clinical signs, viral anti-
gen was only found in lymphoid tissues and epitheli-
um of the upper portion of the digestive tract.

Within individual tissues, distinct patterns of viral 
spread were observed in our study. In sites with a 
mucosal lining, viral antigen was detected first in 
mononuclear cells in the lamina propria and multi-
 focially in the epithelium, then diffusely in the epithelium 
and lamina propria. From the mucosa, the virus spread 
to the lamina muscularis mucosae, submucosa, and 
tunica muscularis. As reported, viral antigen was also 
detected in a few intramural ganglia. A comparable dis-
tribution in the intestinal mucosa was not seen after 
infection with other BVDV 2 strains that generally 
infected the epithelium of the upper portion of the 
digestive tract.

Comparing the endocrine tissues, the pancreas 
was affected especially early, with viral antigen first 
detectable in the islets and later also in the glan-
dular epithelium. In the adrenal and pituitary glands, 
the neuronal portions (adrenal medulla and neurohypoph-
ysis) were initially and more severely infected than 
non-neuronal portions. Despite its wide distribution, 
viral antigen was inconsistently found in the skin, even 
in advanced infection. This confirms that skin biopsy 
specimens are not reliable for diagnosis of acute BVDV 
infection. In the liver, kidney, testes, and heart, viral 
antigen was initially found in vascular walls and inter-
stitial connective tissue. Viral antigen remained 
restricted to these sites in the liver, whereas it spread to 
the surrounding parenchymal cells in the kidney. The 
importance of BVDV antigen in vascular walls has been 
questioned repeatedly, but infection of vascular walls 
was consistently detected in calves with acute infection 
and widespread distribution of virus in our study.

These findings indicate that there is no restriction 
for entry and replication of noncytopathic BVDV in 
certain tissue types, and that the virus has the ability to 
replicate in all cell types throughout the body. This is 
in accordance with observations in calves born persist-
ently infected with BVDV. The pattern of viral 
spread to different organs is most likely related to the 
pathways and preferential recirculation patterns of the 
cells that initially become infected in the lymphoid tis-
ues, such as lymphocytes, because only lymphocytes 
are capable of circulating between blood and lymphoid 
tissues. In the late phase of infection, viral antigen in 
vascular walls might also contribute to the pattern of 
spreading.

In all tissues examined, viral antigen was initially 
detected without associated lesions. Tissue alterations, 
when detected, developed later. In the lymphoid tis-
sues, there was good correlation between distribution of viral antigen and lesions. Lesions characterized by increased apoptosis and depletion of lymphocytes were detected in all compartments where viral antigen was detected. This included lymphoid follicles and T-cell-dependent areas of tonsils, lymph nodes, gut-associated lymphoid tissues, spleen, and thymus. The destruction of lymphocytes was reflected in the severely reduced number of circulating lymphocytes.

The correlation between distribution of viral antigen and lesions in the mucosa of the digestive tract was not quite as clear. Increased numbers of apoptotic cells in the proliferative compartments were detected at sites with viral antigen, but lesions such as erosions or crypt hypoplasia developed multifocally and did not match the diffuse distribution of viral antigen. Comparable lesions diagnosed as intestinal crypt epithelial cell necrosis have been consistently reported in cases of acute BVDV 2 infection,17,19-21 even in animals in which no viral antigen could be detected in the intestinal mucosa.22 In those investigations, a better correlation was observed between distribution of viral antigen and necrosis of epithelium in the upper portion of the digestive tract.17,20,21

Consistent with other reports17,22 no difference in the amount and distribution of viral antigen was found between inflamed and normal pulmonary tissue, indicating no direct virus-mediated damage. On the basis of the observation that bacterial pathogens are regularly isolated from altered lung tissues,22,23 it is highly likely that pulmonary infections are caused by generalized immune suppression. Despite the presence of viral antigen, no lesions were observed in several organs (liver, kidney, heart, endocrine organs) and tissues (vascular walls, smooth and striated muscle, neurons).

The development of lesions might be explained by differences in the susceptibility of infected cells to changes of the cellular metabolism induced by the replication of noncytopathic BVDV. As the term noncytopathic indicates, noncytopathic strains of BVDV do not induce apoptosis in epithelial cell culture,19,24,25 but they cause changes in basic metabolic activity of peripheral blood mononuclear cells,26 to which highly proliferating cells might be especially susceptible. This effect might be augmented by high amounts of viral antigen and thus increase with time after infection. Conversely, delayed development of lesions might also indicate involvement of the host's immune system. This hypothesis is supported by the finding that the older calves, which have a more mature immune system,27 developed more severe clinical signs and lesions.

The importance of the individual host's reaction was emphasized by finding that 1 calf was able to clear the virus from most tissues. Virus isolation confirmed that the calf had been successfully infected, and histologic examination revealed various degrees of depletion and recovery of its lymphoid tissues. Despite clearance from many organs and tissues, viral antigen persisted in arterial walls and was associated with severe inflammation. These lesions may have been immune-mediated, because comparable vascular changes can develop after precipitation of immune complexes. Similar lesions have been reported in the ileal submucosa and at the hilus of the retropharyngeal and bronchial lymph node after inoculation with BVDV 221 and in adult cattle with late onset mucosal disease.36

References


Correction: Use of noninvasive dental dolorimetry to evaluate analgesic effects of intravenous and intrathecal administration of morphine in anesthetized dogs.

In the article "Use of noninvasive dental dolorimetry to evaluate analgesic effects of intravenous and intrathecal administration of morphine in anesthetized dogs." (AJVR, Oct 2002, pp 1349-1353), the reference number 49 should appear as follows:


Correction: Cutaneous analgesia, hemodynamic and respiratory effects, and β-endorphin concentration in spinal fluid and plasma of horses after acupuncture and electroacupuncture.

In the article “Cutaneous analgesia, hemodynamic and respiratory effects, and β-endorphin concentration in spinal fluid and plasma of horses after acupuncture and electroacupuncture.” (AJVR, Oct 2002, pp 1433-1442), there were 2 footnotes listed as “k”. The footnotes should appear as follows:

k1ITO IC 4107, M.E.D. Servi-Systems Canada LTD, Stittsville, ON, Canada.
k2ABL 500-K pH and blood gas analyzer, Radiometer-Copenhagen, Copenhagen, Denmark.
k3Criticaps, micro-hematocrit capillary tube reader, Monojet Scientific, St Louis, Mo.
k410436 Veterinary refractometer, Cambridge Instruments, Buffalo, NY.