Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Experimental Superinfection of Bovine Viral Diarrhea Virus in Persistently Infected Korean Native Calves

Ha-Young Kim[1,§], Eun-Jung Bak[2,§], Jae-Jo Kim[1] and Gye-Hyeong Woo[3,*]

[1]Animal Disease Diagnostic Division, Animal and Plant Quarantine Agency, Gimcheon, Gyeongbuk 39660; [2]Oral Cancer Research Institute, Yonsei University, Seoul 03722; [3]Laboratory of Histopathology, Department of Clinical Laboratory Science, Semyung University, Jecheon, Chungbuk 27136, Republic of Korea
*Corresponding author: ghwoo@naver.com

ARTICLE HISTORY (15-289)

Received: June 07, 2015
Revised: February 03, 2016
Accepted: March 28, 2016
Published online: June 29, 2016

Key words:
BVDV
Cattle
Inoculation route
Mucosal disease
Persistent infection

ABSTRACT

Two male calves infected with non-cytopathic (ncp) bovine viral diarrhea virus (BVDV) BVDV-1c in survey study were donated and superinfected experimentally with cytopathic (cp) BVDV-1a via the intranasal (calf 1) or intravenous (calf 2) route to perform clinical, pathological, and serological evaluation using different inoculation routes. Both calves were moribund and euthanized at post-inoculation days (PID) 30 and 38 without diarrhea, respectively. Gross and histopathological lesions were not prominent in calf 1, and the cp BVDV could not be isolated from samples from this calf. More severe and characteristic upper gastrointestinal lesions, including deep ulceration and lymphocytic inflammation in the digestive system, were noted in calf 2 than in calf 1. Moreover, the cp BVDV-1 was isolated from various organs or tissues in calf 2. These results indicate that the inoculation route of cp BVDV-1 could be an important factor in the induction of mucosal disease in PI calves.

To Cite This Article: Kim HY, Bak EJ, Kim JJ and Woo GH, 2016. Experimental superinfection of bovine viral diarrhea virus in persistently infected Korean native calves. Pak Vet J, 36(3): 338-343.

INTRODUCTION

Bovine viral diarrhea virus (BVDV), a *pestivirus* of the Flaviviridae family, is an economically important cattle pathogen with a worldwide distribution (Peterhans *et al.*, 2003; Becher and Thiel, 2011). Infection with BVDV is associated with a wide range of clinical symptoms, including reproductive problems, enteritis and pneumonia in calves, and the occurrence of fatal mucosal disease (MD) (Evermann and Barrington, 2005; Lanyon *et al*., 2014). MD is characterized by fatal watery-bloody diarrhea, ulceration of the gastrointestinal tract, necrosis and depletion of lymphoid tissues, leukopenia and immunosuppression (Loehr *et al.*, 1998; Chase, 2013).

BVDV has been divided into two genomic subsets, type 1 and 2, and into two biotypes, cytopathic (cp) and noncytopathic (ncp) strains, based on viral cytopathic effects in infected cell monolayers (Gamlen *et al*., 2010). Infection of the bovine fetus with ncp BVDV before the formation of immune system causes viremia in the fetus that can persist into adult life (Peterhans and Schweizer, 2013). Persistently infected (PI) animals are at high risk for developing MD (Ridpath *et al*., 2015). Severe-to-fatal BVDV-mucosal disease (BVDV-MD) noted with early-onset MD and late-onset MD, is believed to result from superinfection with a cp BVDV in PI cattle. The cp BVDV might have been exogenous, originating from other cattle, or endogenous, originating from mutation of the ncp BVDV involved in the persistent infection (Wilhelmsen *et al*., 1991). The cp BVDV strain, however, does not always induce MD in persistently viremic cattle, and the immunotolerant cattle may respond to BVDV strains that are different antigenically from the PI strain (Loehr *et al*., 1998; Sentsui *et al*., 2001; Aduriz *et al*., 2015).

The inoculation route could affect the results of experimental infection, and the route could be changed due to organ tropism of the virus, such as neurotropism or nephrotropism (Hirano *et al*., 2004). Many reports have suggested that the intravenous (IV) route is more effective to induce diseases and significantly causes higher mortality than the intranasal (IN) route of inoculation (Rhoades and Rimler, 1993; Sarver *et al*., 2005).

Previous MD induction studies have been performed according to different antigenic relationships, immunologic factors and/or organ tropisms (Wilhelmsen *et al*., 1991; Loehr *et al*., 1998; Sentsui *et al*., 2001). However, clinical,

§These Authors contributed equally to this work.

pathological, and serological changes using different inoculation routes have not been previously evaluated in PI calves with BVDV. In the present study, we used two donated naturally PI calves through survey studies and experimentally inoculated them with cp BVDV-1 using the IN or IV route, respectively, and compared the induction of MD according to the two inoculation routes.

MATERIALS AND METHODS

Animals: Two 8-month-old male Korean native calves were acquired from the same farm through previous survey studies (Bae *et al*., 2007). The viruses isolated in calves used in the present study were ncp BVDV-1c strains. Both calves tested negative for BVDV-specific antibody prior to the start of the experiment (Table 1). All animals were clinically examined prior to inoculation and were examined daily for the duration of the experiment. Calf 1 had mild respiratory signs with growth retardation and a rough hair coat, and calf 2 had mild lameness with growth retardation and a rough hair coat. Except for these signs, the calves were clinically healthy with no apparent developmental abnormality. The calves were treated in accordance with the guidelines for animal experimentation of the Animal and Plant Quarantine Agency.

Experimental procedure: Calves were kept in isolated facilities with *ad libitum* access to water, pelleted feed and hay. BVDV isolate 946 (BVDV type 1a) as heterologous challenge strain, was prepared for inoculation of PI cattle. After 2 weeks of adaptation, calf 1 was infected with $10^{7.0}$ tissue culture infective dose $(TCID)_{50}$/mL of the 946 strain of cp BVDV-1 in 10 mL tissue culture fluid (5 mL/nostril) intranasally, and calf 2 was also infected with the same virus in 10 mL tissue culture fluid intravenously via the jugular vein. Calves were monitored twice daily for clinical signs and rectal temperatures. For humane reasons such as feed ingestion difficulty due to astasia and pain, calves were anesthetized, exsanguinated, and necropsied at PID 29 and 37, respectively.

Histopathology and immunohistochemistry: All samples were fixed in 10% neutral-buffered formalin, embedded, sectioned, and stained with hematoxylin and eosin (HE).

For the detection of viral antigens, all of the slides were treated with 0.1% protease XIV (Sigma-Aldrich, St. Louis, MO, USA) for 10 min at 37°C and then incubated with protein blocked serum-free solution for 10 min at room temperature (RT) to saturate nonspecific protein-binding sites. The slides were incubated with primary monoclonal antibody (15C5; 1:1000; IDEXX Laboratory Inc., Westbrook, ME, USA) for 30 min at RT in a humid chamber and then incubated for 20 min at RT with biotinylated link antibody (Dako North America, Inc., Carpinteria, CA, USA) and streptavidin-AP (Dako North America, Inc.), respectively. The final reaction was produced by immersing the sections in a solution of red chromogen (Dako North America, Inc.) for 10 min at RT. The sections were counterstained with Mayer's hematoxylin and mounted for microscopy.

RNA extraction and RT-PCR: RNA was extracted from blood, nasal and saliva swabs, and fecal samples using the RNeasy Miniprep kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. First-strand cDNA synthesis and PCR amplification was conducted using the One-step RT-PCR kit (Qiagen). For nested PCR, the Taq PCR Master Mix Kit (Qiagen) and primer sets (Bae *et al*., 2007) were used.

Virus isolation: Viruses were isolated from the organs and tissues of euthanized animals using MDBK cells. After two additional blind passages, the plates were examined to determine the CPE of cells by low-magnification microscopy at each passage.

Hematological analysis: Peripheral blood samples were collected from all of the calves every 3 or 4 days using sodium heparinized tubes. Complete blood cell counts were measured using an automatic cell counter (Hemavet 950; Drew Scientific, Dusseldorf, Germany).

Neutralizing antibody test: To detect BVDV-specific neutralizing antibodies, 50 µL inactivated sera was serially diluted two-fold in 50 µL alpha MEM. A cytotoxic BVDV suspension (100 $TCID_{50}$/ml) was added to the wells and then incubated for 3–4 days in an incubator containing 5% CO_2. To read the SN titer, CPE patterns of cells were observed under low-magnification microscopy.

RESULTS

Clinical findings: Calf 1 and calf 2 showed slight febrile reactions (40.5°C) on PID 4-11 and PID 3–7, with peak fever reaching 40.6°C and 40.8°C, respectively. Those results were concordant with the period of inappetence (data not shown). Although the rectal temperature varied considerably, a clear difference was evident between before and after inoculation. After superinfection, both animals showed pyrexia and anorexia as mentioned, but diarrhea was not observed until both calves were euthanized for necropsy. Calf 1 became suddenly recumbent on PID 28 with respiratory signs, and calf 2 became recumbent on PID 35 with no MD-like clinical symptoms.

Complete blood counts: No significant change was noted in the number of total white blood cells and packed cell volume throughout the experiment in either calf. However, moderate lymphocytopenia was detected from PID 5-12 in calf 2 (data not shown).

Gross findings: Gross lesions were not prominent in calf 1, which had dark-red, pinpointed spots on the surface of the kidney and failure of lung collapse. In calf 2, multifocal characteristic erosions and ulcers were scattered in the digestive tract, suggesting a typical lesion of MD. These erosions and ulcers were present in the oral mucosa, tongue, and abomasal mucosa (Fig. 1). Multifocal red spots were observed in the small intestine, gall bladder, and kidneys. Retropharyngeal lymph nodes were enlarged.

Histopathologic findings: In calf 1, no significant MD-like lesion was detected in the GI tract, but few lymphoplasmocytic cells infiltrated into the mucosal layer of the abomasum (Fig. 2) and intestine, and the white pulp of the spleen was mild atrophic. In addition, severe bronchointerstitial pneumonia was observed.

Table 1: Experimental production of MD in PI calves infected with ncp BVDV-1 by different routes of inoculation of cp BVDV

Calves	Route of inoculation	Presence of ncp BVDV at pre-inoculation	Neutralizing antibodies to BVDV-1		Histopathologicmucosal disease-like lesions	cp BVDV isolation at postinoculation
			Pre-inoculation	Post-inoculation		
Calf 1	Intranasal	+	-	+ *	-	-
Calf 2	Intravenous	+	-	+ #	mild	+

*, Start to develop at PID 9; #, start to develop at PID 5.

Table 2: Distribution of viral antigen in tissues after inoculation with cp BVDV-1

Tissue/ Organ	Calf 1		Calf 2	
	Intensity	Antigen distribution	Intensity	Antigen distribution
Central nervous system				
Cerebrum	++	Neuron, endothelial cells	++	Neuron
Cerebellum	-		++	Neuron
Brain stem	+	Neuron	++	Neuron
Digestive system				
Tongue	+	Fibrocytes	+	Vascular endothelial cells
Esophagus	+	Epithelial cells	+	Epithelial cells, fibrocytes
Forestomach	+	Lamina propria	++~+++	Epithelial cells, fibrocytes
Abomasum	+	Lamina propria	+++	Glandular epithelial cells,
Small intestine	-		++	Peyer's patch (macrophages) in the lamina propria and submucosa
Large intestine	-		+++	Mucosa to serosa
Respiratory system				
Trachea	+	Epithelial cells, fibrocytes	++	Fibrocytes, endothelial cells
Lung	+	Alveolar macrophage Bronchial epithelial cells	+++	Macrophages and PMNs Bronchial epithelial cells
Lymphoid system				
Tonsil	++	Crypt epithelial cell Lymphatic nodule	+++	Crypt epithelial cells, lymphocytes and macrophages in Lymphatic nodule
Spleen	+++	Macrophages around white pulp	++	Macrophages around white pulp
Lymph nodes	+++	Medullary macrophages	+++	Medullay macrophages
Urogenital system				
Kidney	+	Tubular epithelial cells	+	Tubular epithelial cells
Urinary bladder	+	Myocytes	++	Epithelial cells, fibrocytes
Endocrine system				
Thyroid gland	++	Follicular cells, parafollicular cells	++	Follicular cells, parafollicular cells
Pancreas	+	Langerhans islet, acinar cells	+	Langerhans islet, acinar cells
Others				
Heart	-		+	Vascular endothelial cells
Liver	++	Sinusoid, Kupffer cells	+	Sinusoid, PMNs
Skin	+	Keratinocytes, follicular cells	++	Keratinocytes, follicular cells

* The intensity of immunostaining and number of BVDV antigen-containing cells: -, no staining; +, faint minimal staining; ++, moderate staining; +++, intense staining; PMNs, polymorphonuclear cells.

Table 3: Results of RT-PCR and the CPE after three passages in organs/tissues at necropsy

System	Organ or tissue	Calf 1		Calf 2	
		PCR_T1	CPE after P3	PCR_T1#	CPE after P3
Nervous	Cerebrum	+	-	+	-
	Cerebellum	+	-	+	-
Digestive	Oral mucosa	+	-	±	-
	Tongue	-	-	+	-
	Esophagus	+	-	±	+
	Rumen	+	-	+	-
	Reticulum	+	-	+	-
	Omasum	+	-	+	-
	Abomasum	+	-	+	++
	Small intestine	+	-	+	++
	Large intestine	+	-	±	-
Respiratory	Trachea	+	-	+	-
	Lung	+	-	+	++
Lymphatic	Thymus	+	-	±	-
	Tonsil	+	-	+	-
	Spleen	+	-	±	++
	Bone marrow	+	-	+	-
	Lymph nodes	+	-	+	++
Urinary	Kidney	+	-	+	++
	Urinary bladder	+	-	+	++
Endocrine	Adrenal gland	+	-	±	++
	Thyroid gland	+	-	+	++
	Pancreas	+	-	-	-
Others	Heart	+	-	-	-
	Liver	+	-	-	-
	Ear skin	+	-	+	-

PCR_T1, PCR for BVDV type 1; P3, three passages; #, RT-PCR result after three passages was positive in organs or tissues; -, negative; ±, weakly positive; +, positive; ++, strongly positive.

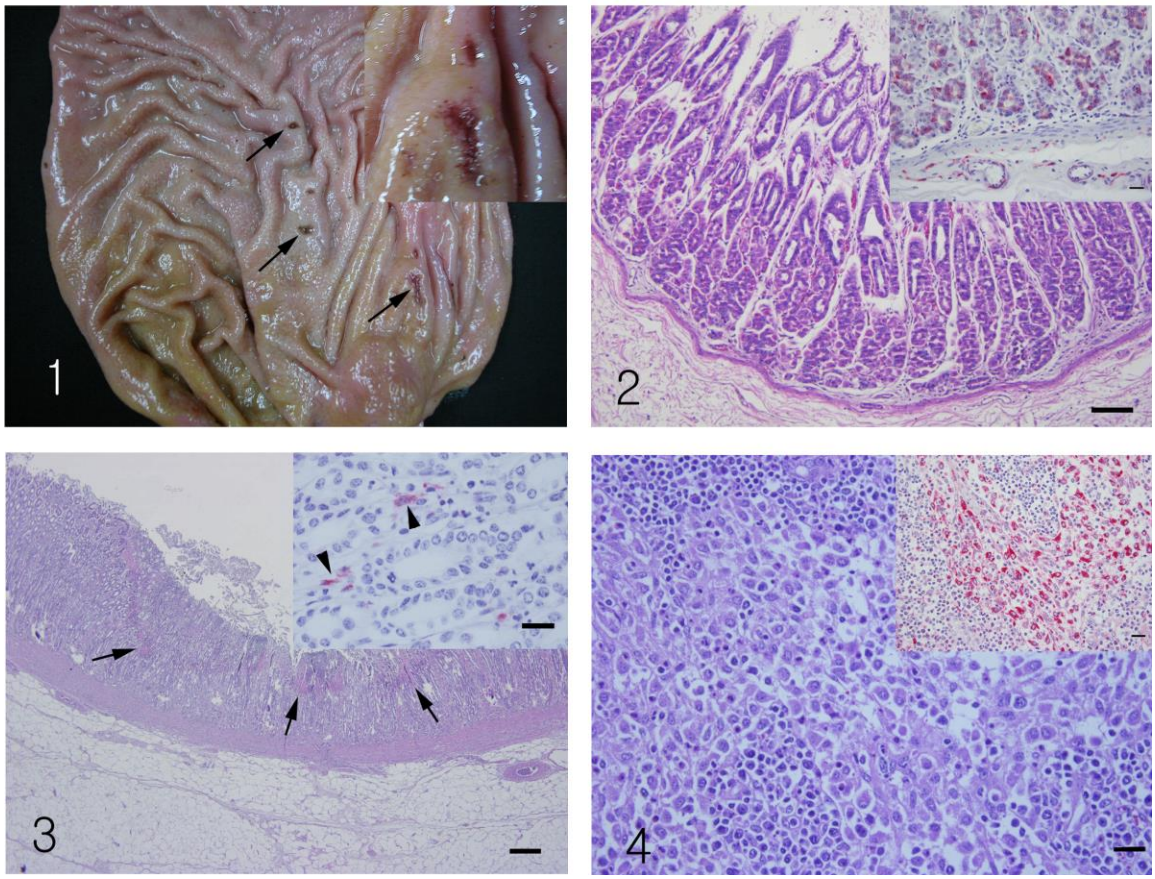

Fig. 1: Abomasum. Erosion and ulcer (arrows) were observed in calf 2 inoculated intravenously with BVDV-1. *Insert:* Close up of erosion and ulcer; **Fig. 2.** Abomasum. Fundic glandular region of calf 1 inoculated intranasally with BVDV-1. Few lymphoplasmocytic cells were infiltrated into the mucosa. HE. Bar = 200 µm. *Insert:* Note the positive reaction in the glandular epithelial cells and vascular walls. IHC. Bar = 50 µm; **Fig. 3.** Abomasum. Erosion was observed in the abomasal mucosa of calf 2. Few degenerative neutrophils and mononuclear cells infiltrated into the erosive lesion (arrows). HE. Bar = 500 µm. *Insert:* Note the immunoreaction (arrow heads) in the erosive lesion of the cells. IHC. Bar = 50 µm**. Fig. 4.** Lymph node. Lymphoid depletion was observed in calf 2. HE. *Insert:* Note the specific reaction of BVDV antigens within macrophages of medullary sinuses. IHC. Bar = 50 µm.

More severe gastrointestinal lesions were found in calf 2 than in calf 1. Deep ulceration into the submucosal layer and lymphocytic inflammation were observed in the tongue. Erosions and ulcers of the abomasal mucosa (Fig. 3) and submucosal hemorrhage in the small intestine were also observed. Furthermore, lymphoid depletion was noted in several lymph nodes (Fig. 4). In addition to those lesions, interstitial nephritis with multifocal hemorrhage, epicarditis, myocarditis, and mild encephalitis were observed. However, no significant respiratory lesion, except mild bronchus-associated lymphoid tissue hyperplasia and very mild lymphocytic inflammation around blood vessels, was observed.

Detection and tissue distribution of BVDV antigen: The IHC results are shown in Table 2. Immunoreactivity was present in the cytoplasm of epithelial cells and neurons without background staining (Inserts of Figs. 2, 3 and 4). The positive reactions were closely related to histopathological lesions and were stronger in calf 2 than in calf 1.

Serology: No serum-neutralizing (SN) antibodies to cp BVDV-1 were detected in either calf prior to experimental infection. SN antibodies against the inoculated cp BVDV-1 were initially detected at PID 9 and PID 5 in calf 1 and calf 2, with titers reaching 2^{10} and $2^{11.5}$, respectively. Calf 2, inoculated intravenously, developed peak neutralizing antibody titers on PID 12, while calf 1, inoculated intranasally, developed peak neutralizing antibody titers on PID 23 (data not shown).

RT-PCR and virus isolation: Through regular sampling of feces, blood, nasal discharge and saliva from both calves, the BVDV-1 was consistently present, according to RT-PCR. The RT-PCR results from tissues/organs are shown in Table 3 and were similar to those of histopathological lesions and IHC. Additionally, other viruses involved in bovine diarrhea, including bovine coronavirus and bovine rotavirus, were not detected in the fecal samples.

BVDV-1 with a CPE after three passages was isolated from various organs of calf 2, including the digestive tracts. However, the virus was not isolated from any organ in calf 1 (Table 3), suggesting that the virus may be neutralized by antibodies or may proliferate slowly after infection.

DISCUSSION

Different routes of inoculation could affect the induction of experimental infection (Hirano *et al*., 2004; Sarver *et al*., 2005). Experimental infections by some viruses, such as avian influenza, have only been successfully induced through selected routes of inoculation

(Vascellari *et al*., 2007). Most previous studies on the induction of MD have shown that both IN and/or IV inoculation routes were widely used, and MD was developed successfully between PID 9 and 24 (Bezek *et al*., 1995; Semrau *et al*., 2008). In the present study, erosions and ulcers were observed in the digestive tracts of calves injected intravenously, although diarrhea was not observed.

MD occurs after infection of a PI animal with a cp BVDV strain that is antigenically homologous to the persisting ncp strain (Nakajima *et al*., 1993). However, the potential virulence and organ-tropism of the strain should be the primary focus (Sentsui *et al*., 2001). The tropism of BVDV to lymphoid tissue and enterotropism of BVDV isolates have been documented (Wilhelmsen *et al*., 1991). Furthermore, some results have indicated that the antigenic similarities were not sufficient to predict the outcome of MD (Loehr *et al*., 1998; Aduriz *et al*., 2015), especially when cellular immunity was suppressed (Sentsui *et al*., 2001). In the present study, only calf 2 inoculated by the IV route following superinfection with the antigenically heterologous cp BVDV-1 showed MD-like pathological lesions, and a cp virus was isolated from the same calf. Even in calf 2, the MD-like lesions were limited to digestive organs, and the calf showed only pyrexia and anorexia. The distribution of antigens was consistent with that previously reported for BVDV infection (Njaa *et al*., 2000; Pedrera *et al*., 2012). Although we could not distinguish cp BVDV from ncp BVDV via RT-PCR or IHC, the cp BVDV was isolated by cell culture according to histopathological lesions in calf 2. Through these results, we considered that the challenge strain on its own had too low of a virulence to induce clinical signs of MD, or the viral affinity for epithelial cell was decreased due to cell adaptation. Therefore, the IV route could be more effective for inducing MD-like lesions in PI cattle if the pathogenesis of isolates as the challenge strain has not yet been reported as in the present study. Rhoades and Rimler (1993) reported that a higher mortality was caused when even the less virulent *Pasteurella multocida* was administered intravenously than orally.

PI cattle have been reported to display growth retardation and immunosuppression and increased frequency of secondary infections (Peterhans *et al*., 2003; Yamane *et al*., 2008). In the present study, animals showed growth retardation and a rough hair coat before superinfection. Calf 1 contracted pneumonia by bacterial infection. However, digestive problems, such as loss of appetite and diarrhea, were not observed prior to challenge, suggesting that these calves might not have contracted other secondary infections before superinfection. Additionally, viral antigens such as rotavirus and coronavirus were not detected, using PCR, in feces before superinfection.

Acute BVDV infections induce transient immunosuppression involving leukopenia along with direct infection of lymphocytes and macrophages because of the virus' predilection for cells that play roles in the host immune system (Evermann and Barrington, 2005). Additionally, these infections have major effects on T-lymphocytes and B-lymphocytes and often result in a severe reduction in the circulating number of lymphocytes, as well as suppression of the functional activities of these cells. Granulocytes and monocytes are equally susceptible to BVDV infection with reduction in numbers and suppression of functions (Brewoo *et al*., 2007). Our finding showed that moderate, transient lymphocytopenia was detected in calf 2, suggesting that intravenous inoculation of BVDV could induce moderate lymphocytopenia.

During the development of MD, two patterns are noted—early-onset MD and late-onset MD. The animals with early-onset MD become moribund within 2–3 weeks after superinfection; those with late-onset MD recover after the transient phase of mild disease and develop MD after a period varying from 4 weeks to several years, eventually leading to death of the animal (Loehr *et al*., 1998). In previous studies, antigenically heterologous BVDV strains have been considered to be the cause of late-onset MD (Fritzemeier *et al*, 1997), and this restricted homology has been documented to hinder early development of MD (Semrau *et al*., 2008). These animals can also develop antibodies against BVDV, as in our study. In the present study, moderate to high levels of neutralizing antibodies against the inoculated cp strains were found in both animals, indicating that calves became infected with the heterologous strain. Additionally, the absence of diarrhea in combination with the presence of lesions in the tongue, abomasum and lymph nodes of calf 2 might indicate either recovery from the infection by cp BVDV or the start of late-onset MD.

Conclusions: Mild but characteristic histological MD-like lesions were observed in PI calf 2 infected intravenously with cp BVDV-1. Although viral antigens were detected in affected organs or tissues of both animals by IHC, cp BVDV-1 was isolated from organ/tissue samples of calf 2 only. Therefore, not only the virulence, antigenic relationship, immunologic factors, and challenge dosage, but also the inoculation route could be an important factor in the experimental induction of MD in PI calves. To our knowledge, ours is the first report to compare the induction of MD via different inoculation routes using the BVDV isolated in Korea.

Acknowledgments: We thank Mr. Jung-Won Park for help with histotechnology.

Author's contribution: HYK undertook the experimental work including animal experiment and sampling, EJB and GHW analyzed all experimental data including histopathological data, JJK executed virological experiment, and GHW designed the experiment. HYK, EJB and GHW wrote the manuscript. All authors read and approved the final manuscript.

REFERENCES

Aduriz G, Atxaerandio R and Cortabarria N, 2015. First detection of bovine viral diarrhoea virus type 2 in cattle in Spain. Vet Rec Open, 2: e000110. doi: 10.1136/vetreco-2014-000110.

Bae YC, Kim HY, Park JW, Yoon SS, Woo GH *et al*., 2007. Prevalence for persistent infection with bovine viral diarrhea virus in Korean native calves. Korean J Vet Res, 47: 163-167.

Becher P and Thiel HJ, 2011. Pestivirus (Flaviviridae). In: Springer index of viruses (Tidona C and G Darai, eds). 2nd Ed, Springer Velag, Heidelberg, Germany, pp: 483-488.

Bezek DM, Stoffregen D and Posso M, 1995. Effect of cytopathic bovine viral diarrhea virus (BVDV) superinfection on viral antigen association with platelets, viremia, and specific antibody levels in

two heifers persistently infected with noncytopathic BVDV. J Vet Diagn Invest, 7: 395-397.

Brewoo JN, Haase CJ, Sharp P and Schultz RD, 2007. Leukocyte profile of cattle persistently infected with bovine viral diarrhea virus. Vet Immunol Immunopathol, 115: 369-374.

Chase CC, 2013. The impact of BVDV infection on adaptive immunity. Biologicals, 41: 52-60.

Evermann JF and Barrington GM, 2005. Clinical features. In: Bovine viral diarrhea virus: Diagnosis, management and control (Goyal SM and JF Ridpath, eds): Blackwell Publishing, Oxford, UK, pp: 105-120.

Fritzemeier J, Haas L, Liebler E, Moennig V and Greiser-Wilke I, 1997. The development of early vs. late onset mucosal disease is a consequence of two different pathogenic mechanisms. Arch Virol, 142: 1335-1350.

Gamlen T, Richards KH, Mankouri J, Hudson L, McCauley J *et al*., 2010. Expression of the NS3 protease of cytopathogenic bovine viral diarrhea virus results in the induction of apoptosis but does not block activation of the beta interferon promoter. J Gen Virol, 91: 133-144.

Hirano N, Nomura R, Tawara T and Tohyama K, 2004. Neurotropism of swine haemagglutinating encephalomyelitis virus (Coronavirus) in mice depending upon host age and route of infection. J Comp Path, 130: 58-65.

Lanyon SR, Hill FI, Reichel MP, and Brownlie J, 2014. Bovine viral diarrhoea: pathogenesis and diagnosis. Vet J, 199: 201-209.

Loehr BI, Frey HR, Moennig V and Greiser-Wilke I, 1998. Experimental induction of mucosal disease: consequences of superinfection of persistently infected cattle with different strains of cytopathogenic bovine viral diarrhea virus. Arch Virol, 143: 667-679.

Nakajima N, Fukuyama S, Hirahara T, Takamura K, Okada N *et al*., 1993. Induction of mucosal disease in cattle persistently infected with noncytopathic bovine viral diarrhea-mucosal disease virus by superinfection with cytopathic bovine viral diarrhea-mucosal disease virus. J Vet Med Sci, 55: 67-72.

Njaa BL, Clark EG, Janzen E, Ellis JA and Haines DM, 2000. Diagnosis of persistent bovine viral diarrhea virus infection by immunohistochemical staining of formalin-fixed skin biopsy specimens. J Vet Diagn Invest, 12: 393-399.

Pedrera M, Gómez-Villamandos JC, Risalde MA, Molina V and Sánchez-Cordón PJ, 2012. Characterization of apoptosis pathways (intrinsic and extrinsic) in lymphoid tissues of calves inoculated with non-cytopathic bovine viral diarrhoea virus genotype-1. J Comp Pathol, 146: 30-39.

Peterhans E, Jungi TW and Schweizer M, 2003. BVDV and innate immunity. Biologicals, 31: 107-111.

Peterhans E and Schweizer M, 2013. BVDV: a pestivirus inducing tolerance of the innate immune response. Biologicals, 41: 39-51.

Rhoades KR and Rimler RB, 1993. Pasteurella multocida virulence factors: selection of fowl cholera-inducing and non-inducing strains. Avian Dis, 37: 1071-1073.

Ridpath JF, Bayles DO, Neill JD, Falkenberg SM, Bauermann FV *et al*., 2015. Comparison of the breadth and complexity of bovine viral diarrhea (BVDV) populations circulating in 34 persistently infected cattle generated in one outbreak. Virology, 485: 297-304.

Sarver CF, Morishita TY and Nersessian B, 2005. The effect of route of inoculation and challenge dosage on *Riemerella anatipestifer* infection in pekin ducks (*Anas platyrhynchos*). Avian Dis, 49: 104-107.

Semrau A, Wibbelt G, Hilbe M, Lieckfeld K, Hermes R *et al*., 2008. Experimental superinfection of a Lesser Malayan Mousedeer (*Tragulus Javanicus*) persistently infected with bovine viral diarrhea virus. J Zoo Wildl Med, 39: 1124-127.

Sentsui H, Nishimori T, Kirisawa R and Morooka A, 2001. Mucosal disease induced in cattle persistently infected with bovine viral diarrhea virus by antigenically different cytopathic virus. Arch Virol, 146: 993-1006.

Vascellari M, Granato A, Trevisan L, Basilicata L, Toffan A *et al*., 2007. Pathologic findings of highly pathogenic avian influenza virus A/Duck/Vietnam/12/05 (H5N1) in experimentally infected pekin ducks, based on immunohistochemistry and In situ hybridization. Vet Pathol, 44, 635-642.

Wilhelmsen CL, Bolin SR, Ridpath JF, Cheville NF and Kluge JP, 1991. Lesions and localization of viral antigen in tissues of cattle with experimentally induced or naturally acquired mucosal disease, or with naturally acquired chronic bovine viral diarrhea. Am J Vet Res, 52: 269-275.

Yamane D, Kato K, Tohya Y and Akashi H, 2008. The relationship between the viral RNA level and upregulation of innate immunity in spleen of cattle persistently infected with bovine viral diarrhea virus. Vet Microbiol, 129: 69-79.