Field Distribution of END Phenomenon-Negative Bovine Viral Diarrhea Virus

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ABSTRACT. Field isolates of BVDV which do not show the exaltation of Newcastle disease virus (END) phenomenon (END⁻) are rarely reported. In this study, 45 BVDV field isolates from cattle in Hokkaido prefecture in Japan were analyzed by the reverse plaque formation method, the END method and observation of cytopathic effects. END⁻ virus was detected in 34 of 45 isolates (75.6%), although 35 of 45 field isolates contained END phenomenon positive virus as the predominant virus population. We propose that END⁻ viruses are widely distributed in the field and that it is possible that the mixture of biologically distinct BVDV correlates with the appearance of disease in infected animals.
KEY WORDS: bovine viral diarrhea virus, END phenomenon, pestivirus, quasispecies, reverse plaque formation

Bovine viral diarrhea virus (BVDV) is an enveloped positive-stranded RNA virus that is classified in the *Pestivirus* genus within the family *Flaviviridae*. Infection with BVDV leads to significant economic losses for cattle producers worldwide [8, 28]. Generally, acute BVDV infections cause transient fever and diarrhea, nasal discharge and reduced milk yield, from which animals naturally recover. However, there are also cases where infected cattle show diverse symptoms ranging from clinically inapparent to severe [1, 3]. Moreover, transplacental infection can result in abortion, significant fetal abnormalities or the birth of persistently infected (PI) calves [21]. PI calves continuously excrete large amounts of virus throughout their lives, showing few symptoms, and are a continual source of infection in a herd. Furthermore, they are at risk of developing fatal mucosal disease (MD) [2].

BVDV is classified into cytopathogenic (CP) and noncytopathogenic (NCP) biotypes on the basis of morphological changes in virus-infected cultured cells. Most BVDV field strains are NCP [7], and only NCP virus can establish a persistent infection [5, 9]. A pair of closely related NCP and CP viruses can be isolated from animals that have developed MD. Therefore, it is considered that the appearance of CP virus in a PI animal strongly correlates with the progression to development of MD [4]. Moreover, NCP BVDV and classical swine fever virus (CSFV) can be further divided into two biotypes, one of which shows the exaltation of Newcastle disease virus phenomenon (END⁺) [10, 11, 15] and the other which does not (END⁻) but which interferes with vesicular stomatitis virus (VSV) and western equine encephalitis virus [6, 12, 18, 26]. However, END⁻ virus has been rarely recognized, because most have been isolated from a few virus strains which have been passaged *in vitro* and are detected at a lower ratio than END⁺ phenotypes. Therefore, the epidemiological significance of END⁻ virus in the field is unknown, and it is unclear whether END⁻ virus plays a part in the variation in symptoms of BVDV during infection. Accordingly, we formulated a hypothesis that the ratio of biologically distinct viruses present as quasispecies within the same isolate can influence the clinical condition of an infected host. The purpose of this study was, as an initial step, to detect and quantify BVDV quasispecies in field isolates.

Forty-five BVDV isolates were collected between 2006 and 2009 at three livestock hygiene service centers in Hokkaido prefecture. Epidemiological data about the viral genotype, host and disease status were based on the records collected by the livestock hygiene service centers (Table 1). Although the passage history of each isolate in the livestock hygiene center was unclear, all isolates were propagated in bovine testicular (BT) cells in our laboratory. The supernatants were collected as virus suspensions and used for this study. BT cells prepared from the testes of a BVDV-free calf were grown as monolayers in Eagle's minimum essential medium (MEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% inactivated fetal bovine serum (FBS) and 0.1125% NaHCO₃, 2 mM L-glutamine and antibiotics. FBS was confirmed as being free from pestiviruses and BVDV antibodies by quality control tests [14]. The Miyadera strain of Newcastle disease virus (NDV) and the New Jersey serotype of VSV were used as challenge viruses in the END and reverse plaque formation (RPF) methods, respectively. CP virus and END⁺ viruses in field isolates were quantified by the END method following observation

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Table 1. Profiles of BVDV isolates examined in this research

Isolates	Host				BVDV			
	Breed	Sex	Age (months)	Disease status	Genotype	Biotype	END$^+$ log$_{10}$ (0.69TCID$_{50}$)/ml	END$^-$ log$_{10}$PFU/ml
KM-2	HS	F	1.4	PI	1b	NCP	<1.8	5.0
KM-3	HS	F	19	PI	1b	NCP	4.0	5.0
KM-4	HS	Unknown	Unknown	sPI	1b	NCP	5.6	5.0
KM-5	HS	F	7.9	sPI	2a	NCP	5.6	6.0
KM-6	HS	F	10.7	PI	1c	NCP	6.4	4.5
KM-7	HS	F	39.2	PI	1c	NCP	6.2	<2.0
KM-8	HS	F	0.9	sPI	1b	NCP	<1.8	4.0
KM-9	HS	F	18.3	PI	1b	NCP	5.8	<2.0
KM-10	HS	F	15.3	PI	1b	NCP	5.2	3.8
KM-11	HS	F	24.3	sPI	2a	NCP	4.8	5.0
KM-12	HS	F	3.8	sPI	1a	NCP	6.6	6.5
KM-13	HS	F	3	sPI	1a	NCP	7.0	6.6
KM-14	HS	F	44.6	PI	1a	NCP	5.0	3.3
KM-15	CB	Unknown	0.1	Abnormal labor	1b	NCP	5.6	5.0
NM-1	HS	F	17.6	Unknown	1b	NCP	6.0	5.0
NM-2	CB	M	0.8	Death	1a	NCP	6.6	5.0
NM-3	HS	F	20.6	Death	1b	NCP	6.2	5.2
NM-4	HS	F	23.9	PI	1b	NCP	6.0	<2.0
NM-5	HS	F	23.6	PI	1b	NCP	5.8	4.9
NM-6	HS	F	22.9	PI	1b	NCP	6.6	<2.0
NM-7	HS	F	22.8	PI	1b	NCP	5.8	6.3
NM-8	HS	F	6.1	AI	1a	NCP	6.6	6.4
NM-9	HS	F	9.9	PI	2a	NCP	6.2	4.3
NM-10	HS	F	9.7	PI	2a	NCP	6.4	4.0
NM-11	HS	F	19.3	PI	2a	NCP	6.2	6.0
AB-1	HS	Unknown	Unknown	Aborted fetus	2a	NCP	4.8	4.0
AB-2	HS	F	11.8	sPI	2a	NCP	5.8	4.6
AB-3	HS	F	15.3	sPI	2a	NCP	6.0	3.7
AB-4	HS	F	27.2	sPI	2a	NCP	5.6	5.0
AB-5	HS	F	4.3	PI	1b	NCP	5.2	<2.0
AB-6	HS	F	5.1	sPI	1b	NCP	4.6	4.0
AB-7	HS	F	5.7	sPI	1b	NCP	5.8	<2.0
AB-8	HS	F	10.4	sPI	1b	NCP	6.6	<2.0
AB-9	HS	F	0.1	sPI	2a	NCP	5.2	<2.0
AB-10	HS	F	0.1	sPI	2a	NCP	5.4	<2.0
AB-11	Unknown	Unknown	Unknown	Aborted fetus	1b	NCP	6.0	5.0
AB-12	HS	F	14.5	PI	1b	NCP	6.4	5.0
AB-13	HS	F	19.2	PI	1b	NCP	6.4	5.0
AB-14	HS	F	8.3	PI	1c	NCP	6.2	4.0
AB-15	HS	F	0.3	sPI	1c	NCP	6.2	5.3
AB-16	HS	F	1.8	sPI	1c	NCP	6.6	<2.0
AB-17	HS	F	6.2	PI	1c	NCP	6.8	5.3
AB-18	HS	F	23.6	PI	1a	CP	NT	NT
AB-19	HS	F	3.2	PI	1c	NCP	6.6	4.0
AB-20	HS	F	42.5	PI	1c	NCP	6.4	5.3

HS: Holstein, CB: Crossbred, F: Female, M: Male, PI: Persistent infection, sPI: Suspected of persistent infection, AI: Acute infection, NT: Not tested.

of cytopathic effects (CPE) [10]. Serial 10-fold dilutions of BVDV suspensions were delivered in 50 μl volumes into 5 wells of 96-well plates per virus dilution, and 0.1 ml of medium containing BT cells was added to each well. The cultures were incubated at 37°C in a CO_2 incubator. Five days after inoculation, CPE was observed, and the 50% tissue culture infective dose (TCID$_{50}$) of CP virus was cal-

culated according to the method of Kärber [13]. When CPE was not observed, the culture fluid was aspirated and cells were superinfected with 10^6 TCID$_{50}$ of NDV. The cultures were incubated for a further 3 days. The cells that showed exaltation of CPE by NDV were read as END phenomenon-positive, and the TCID$_{50}$ of END$^+$ virus was calculated. A modified RPF method was used for detection of END$^-$ virus

Table 2. The classification of the analyzed isolates by the composition of quasispecies

Constituent of quasispecies	Region			Total (%)	
	KM	AB	NM		
END$^+$=END$^-$ a)	4	0	3	7	15.6
END$^+$ only and major b)	8	19	8	35	77.8
END$^-$ only	2	0	0	2	4.4
CP c)	0	1	0	1	2.2
Total	14	20	11	45	100

The ratio of quasispecies was calculated by dividing END$^+$ titer by END$^-$ titer. The TCID$_{50}$ of END$^+$ virus was converted into PFU by applying a Poisson distribution. a) END$^+$ titer is twice less than END$^-$ titer. b) Only END$^+$ virus was detected or END$^+$ titer is two to 435 times as many as END$^-$. c) CP virus was detected, but the END and the RPF methods were not tested.

[6, 17–19]. Serial 10-fold dilutions of virus suspension were inoculated onto confluent BT cell monolayers grown in 6-well multiplates. Two wells were inoculated with each dilution and incubated at 37°C for 1 hr before each suspension was aspirated and washed once with PBS. Then, infected cells were covered with 4 ml of overlay medium consisting of 3% methyl cellulose in Eagle's MEM containing 5% FBS and 0.15% NaHCO$_3$. Cells were then held at 37°C in a CO$_2$ incubator for 5 days. The overlay medium was removed by washing with warmed phosphate buffer solution, and each well was reinoculated with VSV at a multiplicity of infection of 2.0 plaque forming units (PFU)/cell and overlaid again with overlay medium consisting of 2% methyl cellulose in Eagle's MEM. After further incubation for 2 days, the cultures were fixed with methanol and stained with 0.2% crystal violet solution. The stained reverse plaques were judged as the cells infected with END$^-$ virus, and the PFU of END$^-$ virus was calculated. The quasispecies ratio was calculated by dividing the END$^+$ titer by END$^-$ titer. The TCID$_{50}$ of END$^+$ titer was converted into PFU by applying a Poisson distribution. For detection and quantification of BVDV regardless of biological properties, the peroxidase-linked assay (PLA) method was performed with anti-NS3 protein monoclonal antibody JCU/BVD/CF10 (TropBio, Queensland, Australia) as the primary antibody and horseradish peroxidase-conjugated sheep antibody to mouse IgG (SurModics, Eden Prairie, MN, U.S.A.) for secondary antibody [20]. The cells that developed a red color in the cytoplasm were judged as BVDV-positive, and the TCID$_{50}$ of BVDV was calculated. Only one isolate (AB-18) was found to contain CP virus, and the titer by CPE observation was 10$^{4.6}$ TCID$_{50}$/ml, and that by PLA was 10$^{5.8}$ TCID$_{50}$/ml. END$^+$ virus was detected in 42 isolates, and the titer ranged from 10$^{4.2}$ to 10$^{7.2}$ TCID$_{50}$/ml (Table 1). These isolates were predicted to contain END$^-$ virus, because interference against NDV was observed in the low dilution zone but there was a clear END effect at higher dilutions, known as zone, phenomenon [26], in 22 of 44 isolates. Interestingly, two isolates (KM-2 and KM-8) did not show any END phenomenon, but pestivirus was detected by PLA at titers of 10$^{5.2}$ TCID$_{50}$/ml and 10$^{4.9}$ TCID$_{50}$/ml, respectively.

The detection of END$^-$ virus by the RPF method was carried out on 44 isolates. END$^-$ virus was detected in 34 isolates at titers ranging from 10$^{3.3}$ to 10$^{6.6}$ PFU/ml (Table 1). END$^-$ virus was also detected in KM-2 and KM-8 isolates at titers of 10$^{5.0}$ and 10$^{4.0}$ PFU/ml, respectively, although the END phenomenon was not observed. These were equivalent to the titers obtained by PLA.

The isolates were divided into four groups based on the titration of their quasispecies (Table 2). The first group of seven isolates (15.6%) contained similar titers of END$^+$ and END$^-$ viruses (END$^+$/END$^-$=0.1 to 2). The second group of 35 isolates (77.8%) consisted of END$^+$ virus only or as a major component (END$^+$/END$^-$>2). The third group of two isolates (4.4%) contained only END$^-$ virus-positivity. The fourth group consisted of a single isolate (2.2%) containing CP virus. These results show that most BVDV field isolates contained END$^-$ virus at various ratios in addition to END$^+$ virus. Furthermore, this investigation revealed that BVDV strains from which virus is not detected by biological tests, such as the END method, also exist in the field. Although there was a report that some CSFV field strains isolated in Thailand did not show the END phenomenon [25] and that reverse plaques were also detected in some CSFV field strains in France [16], a study on the distribution of END$^-$ BVDV in the field has not been conducted before. This is the first report of the distribution of END$^-$ BVDV in the field.

We searched for characteristics relevant to the ratio of quasispecies based on the information for each field isolate obtained from the livestock hygiene service centers. There was no correlation between characteristics, such as age of host animals, viral genotype, disease state, the region where the viruses were isolated and the ratio of quasispecies. However, nine of the isolates from which END$^-$ virus was not detected were derived from asymptomatic infected cows, and one isolate was derived from a cow whose condition was unknown. Nineteen of 45 isolates were derived from asymptomatic infected cows. END$^-$ virus was detected in ten of these, and the component rates with END$^+$ virus differed 275-fold. This result suggests that further investigation, such as recording a host's detailed clinical symptoms, is required to determine whether the population of biologically distinct viruses is involved in the appearance of disease.

It has been reported that the END phenomenon in END$^+$ CSFV and interference with heterologous virus by END$^-$ CSFV are based on the regulation of IFN-α/β by the viral nonstructural protein N^{pro} [22, 24]. Additionally, Ruggli et al. [23] reported that amino acids C$_{112}$ and D$_{136}$ in N^{pro} of CSFV are essential for N^{pro}-mediated degradation of interferon regulatory factor 3. However, a specific amino acid substitution in N^{pro} which distinguishes END$^+$ and END$^-$ viruses has not been reported in BVDV to date. Therefore, sequencing of the N^{pro} region was carried out on isolates from which END$^+$ or END$^-$ virus was not detected, and from which the titers of END$^+$ and END$^-$ viruses were close. Viral RNA was extracted from each BVDV suspension using a High Pure Viral RNA Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. RT-PCR was carried out using a Prime Script RT-PCR kit (TaKaRa Bio,

Otsu, Japan). The N^{pro} of each suspension was amplified and sequenced using primers which were designed from the viral RNA sequence of the NADL strain (accession number M31182). The oligonucleotide sequences and their positions in the BVDV genome were as follows: 277, 5′-AGG GCA TGC CCA AAG CAC ATC TT-3′ (sense: 244–266); 1028, 5′-CCT GGT ATT TGA CTC CAT CTA CCA CTA T-3′ (antisense: 1,046–1,020). After amplification, the PCR products were purified with a High Pure PCR Product Purification Kit (Roche). Direct sequencing of the PCR products was carried out by Operon Biotechnologies (Tokyo, Japan). The nucleotide sequences of N^{pro} were analyzed using GENETYX version 9.0.7 software (Genetyx, Tokyo, Japan).

We determined the amino acid sequence of N^{pro} of 17 isolates classified into groups "END$^+$ = END$^-$", "END$^-$ only" and "END$^+$ only and major" (data not shown). The alignment of these amino acid sequences did not identify a characteristic substitution which related to a detectable amount of quasispecies. This result suggests that biological differences between END$^+$ and END$^-$ virus may not depend on a single amino acid substitution and that other viral genes outside the N^{pro} region may contain the changes that distinguish END$^+$ from END$^-$ viruses.

Taken together, the results of this study revealed that not only BVDV laboratory and CSFV field strains [16, 25], but also BVDV field isolates contain END$^-$ virus at varying ratios. END$^-$ virus is therefore widely distributed in the field. As previously reported, the regulation of type I IFN production by CSFV N^{pro} involves the biological differences between END$^+$ and END$^-$ viruses [23]. Recently, Tamura *et al.* demonstrated that a functional N^{pro} is not the sole virulence determinant, but that the innate immunity regulation by N^{pro} is responsible for the pathogenic differences between the attenuated CSFV vaccine strain GPE$^-$ and highly virulent CSFV strains, such as Eystrup and ALD, in pigs [27]. In this study, BVDV field isolates were found to contain END$^+$ and END$^-$ viruses at various rates. A mixture of biologically distinct BVDV biotypes may be associated with the appearance of disease in infected animals. Further studies are required to investigate the relationship between quasispecies and BVDV infection and to analyze variations in N^{pro} and other viral proteins.

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