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Serological characterization of bovine viral diarrhea virus isolates

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Abstract : Bovine viral diarrhea viruses (BVDVs) were isolated from cattle with respiratory and diarrhea signs as well as persistently infected cattle. These isolates were analysed serologically to characterize serogroups and to compare serological relationship with reference viruses of type Ⅰ and Ⅱ. Most isolates from calf diarrheal cases and persistently infected individuals showed a significant difference in cross-neutralization test with the viruses isolated from nasal discharges showing severe respiratory signs. Serologically most of the commercial vaccine strains could be classified into classical BVDV (type Ⅰ) such as NADL strain. This serological difference among BVDV isolates suggested the need for new vaccines to protect cattle from both respiratory and enteric BVDV infections in field. The immunogenicity of BVDVs which showed a good propagation capability in MDBK cells and high rates of neutralizing activity (isolate : KD26-1, PHG, B5 and 95002) against all viruses used in this study, was confirmed in guinea pig when treated as single or combined groups.

Key words : bovine viral diarrhea virus, cross neutralization, serological comparison, Immunogenicity.

Introduction

Bovine viral diarrhea virus (BVDV) is a pestivirus in Flaviviridae, having an economic importance in cattle industry throughout the world by a wide range of clinical syndromes[1, 2]. Respiratory signs, diarrhea and reproductive failures including abortion and teratogenic defects are general signs of infected cattle. In the case of infection during pregnancy, persistent infection by immune tolerance makes serious herd health problems by disseminating numerous virus particles through nasal discharge and feces as infectious reservoir for the lifetime[2]. Persistent carriers can show mucosal disease and both biotypes (cytopathic(CP) and noncytopathic(NCP)) are then isolated from the animal[3]. Previously this mucosal sign was explained as a superinfection theory with CP strain to the animal infected with NCP strain. Since antigenic similarity of these biotypes is high, mutation theory from CP to

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NCP strain replaced previous theory[4,5] and some recent molecular studies suggested more specific facts such as rearrangement theory on the nonstructural p125 (p54/p80) gene[6,7].

In addition to classical BVDV, a recent study reported a new type of BVDV associated with severe outbreaks and high mortalities in North America[8]. The viruses isolated in these serious cases were relatively different from classical type in genotype by 5' UTR gene analysis and in serogroup by cross-neutralization reactivity using limited number of polyclonal or monoclonal antibody[8~10]. It also was suggested that BVDV type specificity in antibody response to vaccine strains was existing[11]. However, to date, the serological classification of BVDV is not confirmed though some groups reported the difference among viruses based on the results of limited serological comparison.

In Korea, a study reported a case showing serious respiratory and mucosal signs, high-morbidity above 60% and high-mortality above about 30% in infected calves[12]. The features of these viruses were respiratory tropism and serological difference against the viruses isolated from diarrhea and persistent infection. In this study we attempted to characterize the broad ranges of BVD viruses from various clinical signs, geographical distribution and then to classify serotypes.

Materials and Methods

Specimens : Fecal samples and nasal swabs were obtained from clinical cases showing diarrhea, respiratory signs, and persistently infected individuals without clinical signs. Feces were diluted 1 : 10 in α-Minimum Essential Medium (α-MEM, Gibco-BRL, Gaitherburg, USA), clarified by centrifugation at 3,000rpm for 20 min (Sorvall RT6000, Du Pont company, Delaware, USA), and supernatants were filtered, then used for virus isolation and identification. Filterates of nasal swabs diluted in α-MEM were also used as inocula.

Cells and viruses : Madin-Darby bovine kidney (MDBK) cells from ATCC were used for virus isolation and characterization. α-MEM containing 50μg/ml of gentamicin was supplemented with 5% fetal calf serum (FCS, Gibco-BRL) for growth and without FCS for maintenance of the cells after virus inoculation.

Virus isolation : MDBK monolayer in cell culture plates were washed twice with calcium- and magnesium-free PBS, inoculated into 100μl of a filtered sample, and then adsorbed for 1 to 2 hours at 37℃ in 5% CO_2 incubator.

After adsorption, the inocula were discarded, and 1ml of fresh α-MEM containing antibiotics without FCS was added to the cells. The infected cultures were incubated for 7 to 12 days during each passage level, and examined by cytopathic effect (CPE), immunofluorescence antibody (IFA) technique, immunocytochemical staining and RT-PCR.

IFA and Immunocytochemical staining : IFA test was carried out using BVDV monoclonal antibody (MAb) and polyclonal antibody by the procedure described by Murakami *et al* (1983). Immunocytochemical staining using MAb and Vectastain ABC kit (Vector laboratory, Burlingame, CA, USA) was done according to the manufacturer's manual.

Reverse transcriptase-polymerase chain reaction (RT-PCR) : RNA of BVDV was extracted from the infected cell culture supernatant with Ultraspec Ⅱ RNA extraction kit (Biotecx Inc., Houston, USA), and cDNA was synthesized with MLV-reverse transcriptase (Gibco-BRL) using reverse primer. PCR was carried out to amplify BVDV 5'UTR gene with specific primers (forward 5'-GGCTAGCCATGCCCTTAG-3', reverse 5'-GCCTCTGCAGCACCCTAT-3') and *Taq* polymerase (Gibco-BRL) using three temperature steps (denaturation at 94℃ for 1 min, annealing at 50℃ for 45 sec, elongation at 72℃ for 45 sec, 30 cycles), and then the size of amplified DNA products was determined by electrophoresis using 1% agarose gel.

Propagation and cell culture analysis of BVDV isolates : Inoculated cells were observed for cytopathogenicity for seven to twelve days. Virus propagation was analysed by the microtitration method of Reed and Muench[13] after three cycles of freezing and thawing. The virus titer was expressed as reciprocals of the highest virus dilution showing CPE.

Detection of persistent BVDV carriers from herd : To detect persistently infected cattle from herds, all individuals were tested for neutralizing antibody in sera and the individuals showing negative result were analyzed for BVDV

using nasal swabs and feces, and neutralizing antibody. The individuals which showed negative for antibody in dual samples but positive for BVDV by PCR and virus isolation were confirmed as persistently infected carriers.

Production of hyperimmune sera : Hyperimmune sera were raised in guinea pigs by immunization with BVDV concentrates. Each animal was immunized with the purified BVDV mixed with complete Freund's adjuvant (CFA) and then boostered BVDV mixed with incomplete Freund's adjuvant (IFA) at 3 weeks after the first inoculation. The immunized animals were bled at 2 weeks after the second inoculation, and tested for the neutralizing antibody against BVDV.

Immunogenicity test of inactivated viruses : Individual viruses and combined groups of BVDV vaccine candidates were inactivated with binary ethylene imine (BEI), and tested for their immunogenicity and interference between viruses combined using guinea pigs. Each group composed of five heads of animal was inoculated with 1 or 2ml of inactivated viruses intramuscularly. The experimental groups for this test were arranged as nine groups depending on the kinds of viruses and a number of inoculation time.

Virus neutralization (VN) test : Neutralization antibody test against BVDV was performed using MDBK cells in microplates as previously described[10]. VN titer was expressed as reciprocal of the highest serum dilution showing complete inhibition of CPE.

Results

Isolation and propagation of BVDV using MDBK cells : Sixty BVD viruses were isolated from the diarrhea, nasal discharges of cattle shown clinical symptoms associated with BVD, as well as from persistently infected cattle. Most viruses were isolated from calves with severe respiratory signs and diarrhea at the age less than 8 months. Two viruses were detected in adult bulls more than three years during herd check to single out persistently infected individuals (Table 1).

As a result of virus titration, 95037, KS6, A146, KD5, KD26-1, 95002, KSA1, 1889, PHG, H7, B5 and PYR of Korean isolates showed better propagation in MDBK cells than most present vaccine strains or foreign reference viruses as shown in table 2, in addition to the difference of virus propagation patterns in cells among viruses.

Cross-neutralization analysis among isolates and references : The serologic relationship was analysed by cross-neutralization test between forty-three viruses and antisera against twenty BVDVs including Korean isolates and reference strains. The significant diversities in neutralizing titers were observed among viruses. Especially the difference between viruses isolated from nasal swabs and those from diarrhea or persistent infection was bigger than that between any other types (Table 3). The rates of significant neutralization (different more than 4 fold in the cross-neutralization titer between a virus and antiserum against the same virus) against all viruses tested were the highest in an isolate, KD26-1 (62.8%), and a canadian high-pathogenic BVDV, Waters (62.8%). However, the rate of which KD26-1 neutralized significantly other Korean isolates was the higher (72.2 %) than that (63.9 %) of Waters strain, a highly pathogenic canadian reference starain (Table 4). The significant serological differences suggested the imperfect protection against the infection by significantly different viruses in serology.

The evaluation of the potential as a new BVDV vaccine : The selections of virus combinations with the highest cross-neutralization activities were carried out based on the pat-

Table 1. Sources of viruses isolated in this study

Source of specimens	Clinical types			Age of cattle	
	Acute infection		Persistent infection	less than 12 months	more than 12 months
	Diarrhea	Nasal swab			
No. of specimen	37	21	2	53	7

terns and rates of which isolates neutralized significantly all tested viruses as well as propagation titers in MDBK cells. Among Korean isolates, the rate by combination of KD26-1 and PHG was higher than the rates by any other combinations though the rate by the combination of KD26-1 and Waters was higher than that in the case of being compared among all viruses including isolates and references (Table 5). The titers of all selected isolates propagated in MDBK cells were greater by showing more than log titer 6.5 in $TCID_{50}$/ml than that of Waters showing 4.5 Log $TCID_{50}$/ml (Table 2).

Immunogenicity of inactivated combined BVDV vac-

Table 2. Isolation and propagation of BVDV isolates after propagation in MDBK cells

Isolates	CPE*	Titers ($TCID_{50}$/ML)**	Isolates	CPE	Toters ($TCID_{50}$/ml)
32669F	F	3.8	KS6	S	6.5
95037	S	6.0	KS9	S	5.0
32527F	S	5.5	946	S	4.5
945	M	5.0	95049	S	4.5
KD20-1	S	4.5	A146	S	6.0
KD26-1	M	6.5	KD5	M	6.5
95589	F	4.5	KS2-7	M	3.5
KD21-1	F	4.0	SBCV	S	5.5
KSA4	S	1.0	B5	S	7.5
95411	S	5.5	32517F	S	5.5
KD23-1	S	4.2	KS5	S	3.5
96002	F	7.5	KD36-1	S	4.0
95017-1	M	5.0	B3	S	1.0
KSA1	S	6.5	CK207	S	3.5
KD39	F	5.5	CK209	S	5.5
1889	F	7.5	H7	S	6.0
PHG	S	6.5	PYR	S	6.0
CBY	S	5.5			
A (Vaccine strain)	S	6.5	B (Vaccine strain)	F	3.5
C (Vaccine strain)	F	2.5	Sanders	S	3.5
Auburn	S	1.5	SO2	S	3.0
T20	S	2.5	Oregan	S	7.5
Waters	S	4.5	NOSE	S	4.0
Singer	S	5.5	NADL	M	5.0

* F: CPE within 3 to 4 days, M: CPE within 5 to 7 days, S: CPE within 7 to 10 days.
** Virus titers were determined after tenth passages in MDBK cells.

Table 3. Cross-neutralization reactivities among isolates and references

Viruses \ Antisera	KD26-1	Waters	A	C	B	32669F	95037	Singer	A146	PHG	NOSE	B5	NADL	95002	SO2	NY	Oregan	7443	95411	T20
KD26-1	256#	32	128	128	⟩4096	512	⟩4096	⟩4096	⟩4096	8	64	32	64	4	64	32	32	64	⟨2	2
95037	256	32	128	128	2048	512	512	2048	1024	16	128	128	128	64	128	2	4	16	⟨2	4
A146	128	16	64	128	1024	512	256	⟩4096	2048	4	128	128	32	32	64	⟨2	8	512	⟨2	4
95411	64	2	2	16	4	8	4	32	2	64	1024	32	1024	512	2	4	4	2	512	64
32527F	256	32	128	64	2048	256	128	64	1024	8	128	⟨2	32	32	64	4	16	32	⟨2	8
946	256	16	128	128	1024	128	32	32	128	16	512	⟨2	64	32	256	2	4	128	⟨2	2
KD20-1	128	16	64	64	2048	256	256	512	512	16	128	256	64	32	64	4	4	16	⟨2	4
SBCV	128	16	128	64	1024	256	256	1024	512	16	256	32	32	32	128	⟨2	2	64	⟨2	2
KD23-1	128	16	64	64	16	16	64	32	16	8	128	4	64	8	64	2	8	8	⟨2	4
KS5	64	16	64	32	⟩4096	512	128	16	512	16	128	64	32	4	64	⟨2	4	16	⟨2	4
MADL	256	16	64	128	2048	256	128	32	512	16	32	128	512	8	16	4	8	4	2	8
95049	256	128	128	128	512	512	512	32	512	64	1024	8	128	2	128	128	256	16	32	16
KD36-1	128	128	64	64	⟩4096	1024	1024	128	2048	16	256	⟨2	32	16	128	2	8	32	⟨2	8
KD39	128	64	256	64	256	64	32	64	512	32	32	⟨2	16	16	32	2	8	32	⟨2	4
KD5	64	64	512	128	1024	256	128	256	512	32	32	2	32	4	64	4	32	128	⟨2	4
KS6	256	64	64	64	⟩4096	256	128	64	256	16	128	8	64	32	64	64	256	128	64	64
KS9	512	64	256	256	2048	512	128	128	128	32	512	128	128	64	128*	8	32	256	4	8
945	256	128	256	256	256	16	8	256	128	8	256	⟨2	128	32	256	2	4	32	⟨2	2
KD21-1	128	256	64	32	512	64	32	1024	16	8	256	2	32	16	64	4	8	64	2	8
95589	1024	64	256	256	128	32	16	1024	16	8	2048	2	256	256	128	2	4	128	⟨2	8
95017-1	64	64	128	64	1024	512	256	2048	256	32	256	256	32	32	64	4	8	128	2	16
32517F	128	64	64	64	8	32	64	256	8	⟩256	128	128	32	32	64	32	128	16	4	32
B	256	128	128	128	4096*	64	32	⟨2	64	16	256	16	128	32	128	32	32	32	32	64
A	512	64	256	128*	⟩4096	⟩4096	⟩4096	32	2048	NT	512	32	256	⟩4096	128	32	128	64	64	128
C	256	128	16	256	128	64	16	512	32	4	512	⟨2	32	128	1024	8	8	512	⟨2	8
CK209	128	512	32	32	512	128	128	32	256	512	32	4	512	64	16	4	128	1024	8	32
B5	16	64	32	16	1024	128	64	2048	256	128	32	256	16	32	16*	16	16	256	4	32
Nose	8	256	8	8	16	128	512	32	512	⟩256	1024*	256	16	512	⟨2	512	128	64	512	8
Waters	4	128	4	4	32	16	8	64	32	64	⟨2	64	NT	64	⟨2	32	16	32	64	8
1889	16	128	128	8	128	128	16	128	32	⟩256	8	⟨2	4	8	32	4	16	64	⟨2	8
H7	8	128	4	16	16	16	2	64	8	1024	⟨2	32	32	64	⟨2	2	4	32	256	2
PHG	8	128	4	4	32	64	16	32	16	256	4	32	NT	128	8	8	16	32	16	8
PYR	4	128	4	4	64	16	8	64	32	64	⟨2	16	NT	64	⟨2	16	16	32	32	8
CBY	4	128	4	4	64	16	32	64	32	128	2	32	NT	128	⟨2	16	8	64	32	16
Oregan	4	256	8	4	64	64	8	64	64	32	⟨2	32	NT	256	⟨2	64	256*	32	128	32
KS2-7	128	128	32	32	2	64	8	128	32	16	64	⟨2	64	16	64	4	8	128	⟨2	8
32669F	16	128	8	16	8	1024*	4	⟩4096	16	16	32	512	8	32	16	32	64	1024	8	64
Singer	32	128	64	64	2048	256	512	1024	512	16	32	64	NT	64	64	4	16	512	2	8
95002	16	⟨2	8	16	8	4	4	⟨2	4	NT	16	16	64	512	⟨2	16	16	⟨2	512	⟨2
KSA1	32	32	16	64	128	32	8	64	32	512	⟨2	⟨2	128	128	⟨2	4	16	8	⟨2	16
CK207	16	32	8	32	32	1024	1024	64	2048	32	16	256	512	256	4	4	32	8	⟨2	8
T20	8	32	128	8	32	64	16	64	32	32	64	128	NT	32	32	128	64	64	8	256
SO2	⟨2	32	⟨2	⟨2	8	32	64	16	16	32	4	16	NT	16	1024*	8	4	4	32	4

#Virus neutralization titer, The titers of shadowed boxes means significantly different more than 4 fold in the cross-neutralization test between a virus and antiserum against the same virus. Bold box is the cross-neutralization reactivities between the viruses from severe respiratory cases and the antisera against viruses from diarrhea and persistently infected cases.

Table 4. The rates of isolates neutralized significantly among total isolates and references

Classification \ Antiserums	KD26-1	95002	32669F	95037	A146	95411	B5	T20	Singer	NADL	SO2
Neutralizing rates[#] against total viruses (%)	62.8	25.6	41.9	44.2	34.9	11.6	34.9	14.0	34.9	34.2	9.3
Neutralizing rates against Korean isolates (%)	72.2	25.0	44.4	44.4	33.3	8.3	30.5	13.9	38.9	30.6	8.3

Classification \ Antiserums	NOSE	NY	OREGAN	7443	Waters	PHG	B	A	C	VMRD	Rabbit
Neutralizing rates[#] against total viruses (%)	32.6	25.6	20.9	14	62.8	32.6	41.9	60.5	55.5	42.9	23.3
Neutralizing rates against Korean isolates (%)	30.1	19.4	16.7	13.9	63.9	33.3	44.4	63.9	58.3	41.2	25.0

[#] Significantly different more than 4 fold in the cross-neutralization titer between a virus and antiserum against homologous viruses.

Table 5. The selection of combined vaccine candidate strains

Combination	KD26-1+95002	KD26-1+B5	KD26-1+PHG	A+95002	KD26-1+Waters
Neutralizing rates (%)[#]	79.1(86.1)	79.1(80.5)	81.4(86.1)	81.4(83.3)	83.7(91.6)
Propagation in cells	good[##]	good	good	good	good

[#] Neutralizing rates against all viruses used in this study (neutralizing rates against Korean isolates).
[##] Virus titer ($10^{6.5}$~$10^{7.5}$ $TCID_{50}$/ml).

Table 6. The immunogenicity of combined vaccine candidates to guinea pig

Group*	SN virus	3 wks after the first immunization	5 wks after the first immunization	2 wks after the second immunization***
I (KD26-1)	KD26-1	1.6**	1.4	4.2
II (KD26-1+95002)	KD26-1	0.4	2.2	2.8
	95002	4.8	5.4	6.6
III (KD26-1+B5)	KD26-1	1.6	1.8	2.4
	B5	1.2	1.0	6.8
IV (KD26-1+Waters)	KD26-1	1.0	0.0	2.2
	Waters	1.4	1.2	2.0
V (KD26-1+PHG)	KD26-1	1.8	1.6	4.2
	PHG	1.0	2.0	4.0
Control	KD26-1	0.0	0.0	-
	95002	0.0	0.0	-
	B5	0.0	0.0	-
	Waters	0.0	0.0	-
	PHG	0.0	0.0	-

* Volume and titer of inoculation : group I (1 ml, $10^{5.5}$ $TCID_{50}$/ml), group II, III, IV, V (2ml, $10^{5.5}$ $TCID_{50}$/ml)
** Log_2, *** Bled at two weeks after school immunization at three weeks following the first immunization. Each group was assigned with five heads of animal.

cine candidates in guinea pig : The immunogenicity of BVDVs inactivated with BEI was analysed using guinea pig sera bled in term of 3 weeks and 5 weeks sequentially after inoculations. The immunogenicity of viruses showed the difference depending on groups as shown in Table 6. When inactivated KD26-1 was inoculated into guinea pigs, the SN titers were 1.6 in three weeks after first immunization and this level was maintained in five weeks, but when the animals boosted in three weeks after the first immunization showed 4.2 in SN titer. This immunogenicity was reduced when combined with other candidates such as 95002, B5, Waters and PHG. The immunogenicities of individual viruses combined with KD26-1 were higher in groups combined with 95002 and B5 by showing 6.6 and 6.8 Log2 SN titer respectively than any other groups.

Discussion

BVD is a worldwide bovine viral disease which is associated with not only various clinical syndromes including diarrhea, serious respiratory problems, abortion and congenital defects, but also secondary affected diseases such as frequent mastitis or other immunoligically related ailments[14,15]. It has been suggested that BVDV can be classified into serological groups by cross-neutralization reactivities with monoclonal antibodies or polyclonal antibodies and then analyzed serological diversity[16]. And recently, genotyping by sequence analysis on 5' UTR gene was applied to segregate BVDVs from various sources and biotypes. Phylogenic relationship drawn from the sequence homology data elucidated more clear difference between types I and II than the results from serological comparison[8,10]. The variation in pneumopathogenicity among strains of BVDV was reported based on the comparison between different biotypes of BVDV[17].

In this study, we analysed the cross-neutralization reactivities between twenty BVDV antisera and forty-three strains of BVDV isolated from various clinical signs and persistent infection, in addition to reference strains of both type I and II. And these results by serological analysis were compared with pathological tropisms or clinical signs. We could find the significant differences in neutralizing reactivity among strains, especially between strains isolated from diarrhea and severe respiratory signs. From the comparison in rates of viruses neutralized significantly (different more than 4 SN titer between a virus and antiserum against homologous virus) by twenty antisera, strains with high neutralizing rates were chosen as vaccine candidate strains including KD26-1, B5, PHG, Waters and 95002, and compared the immunogenicities and propagation titers when inoculated as single strain or combined with two strains. KD26-1, the isolate from diarrhea, has the highest rate of neutralizing activity of 72.2% against all Korean isolates, followed by waters, the canadian high-pathogenic strain from the individual with respiratory sign, shown neutralizing rate of 63.9%. This difference in neutralizing rates among the viruses implies that Korean isolates may be more effective in protecting from BVDV infections which have been increasingly noticed in Korea.

In the immunogenicity test using guinea pig, the group immunized with KD26-1 reached to 4.2 in SN titer, but combined groups between KD26-1 and other candidate strains including B5, PHG, Waters and 95002 showed the variation among groups. In Group II and III, the antibody titers against KD26-1 were lower than those of group I as 2.8 and 2.4 Log_2 SN titer respectively though the antibody levels against strains, 95002 and B5, combined with KD26-1 reached to 6.6 and 6.8 Log_2 SN titer respectively. This may be explained as a result of interference in animal body between strains combined, which means the combinations between KD26-1 and B5 or 95002 may be less effective in protecting both types of BVDV than group IV and V, the combined groups between KD26-1 and PHG or Waters. However, Waters strain is not a Korean isolate and lower in virus titer when it was propagated in MDBK cells, but neutralizing rates against BVDVs analyzed were not different much with that of KD26-1. These serological differences coincided with genotypes based on sequence homology of 5'UTR gene (Unpublished data).

Most BVD vaccines licensed in Korea are made of a classical type of BVDV (type I) which has lower significant neutralizing rates against BVD isolates in Korea compared to our vaccine candidates. Depending on clinical signs of virus sources, the neutralizing rates were significantly dif-

ferent, especially between group isolated from severe respiratory signs and that from diarrhea.

From these results, we conclude that more than two serological types are present in Korea and the development of new vaccine is required for better protection against heterologous strains. We need further molecular biological studies to characterize strains showing serological heterogeniety.

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