Selection of a Less Pathogenic BVDV Strain for the Construction of Avirulent Chimeric *Pestivirus*

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To select a less pathogenic bovine viral diarrhea virus (BVDV) strain for the construction of chimeric pestivirus harboring classical swine fever virus (CSFV) E2 gene, five Korean BVDV isolates (four type 1 isolates and a type 2 isolate) were evaluated for their pathological and biological properties with respect to porcine infection. Each of five groups of 100-day-old pigs was inoculated intranasally with one of the five BVDV isolates. No clinical sign or leukopenia was observed in any pig throughout the duration of the experiment, but viruses were detected in blood, nasal discharges and postmortem samples using RT-PCR. These results indicated that although the five BVD viruses could infect pigs, they did not cause clinically apparent symptoms. Because of its proper infection dynamics shown in this preliminary animal study and its fast growth rate and quick CPE in cell culture, one isolate (KD26-1) was chosen among the five isolates to test its virulence and immunogenic properties in 40-day-old piglets. Neither clinical sign nor pathological lesion was observed in 40-day-old piglets during the course of infection of isolate KD26-1. The first neutralizing antibodies were detectable 14 days post-inoculation (PI) and increased to 1:128~1:256 28 days PI. A BVDV specific gene was detectable by RT-PCR in tonsil, spleen, inguinal lymph node and brain until 14 days PI. According to this study, it can be concluded that isolate KD26-1 has little pathological effect in pigs and is a candidate for construction of chimeric pestivirus harboring CSFV E2 gene.

Key Words: Bovine viral diarrhea virus, Classical swine fever virus, Animal study, Pigs, Pathological effect

INTRODUCTION

Along with classical swine fever virus (CSFV) and border disease virus (BDV), bovine viral diarrhea virus (BVDV) belongs to the genus *Pestivirus* in the family *Flaviviridae* (1). BVDV is the causative agent of bovine viral diarrhea that is mainly found in cattle, but it can also present itself in pigs while CSFV infects strictly pigs (2~5).

Phylogenic analysis has revealed two genotypes of BVDV, type 1 and type 2 (6). Regardless of genotype, the virus can also be divided into two biotypes, cytopathogenic (CP) or non-cytopathogenic (NCP), depending on the cellular outcome of infection (1, 7~9). Because BVDV and CSFV are of the same genus, their serology is littered with cross-reactions. As a result, pigs infected with BVDV have often misled in-field serosurveillance of CSFV infection (2, 10~12). It has been previously demonstrated that experimental inoculation of BVDV in pigs can interfere with, or even prevent, infection of virulent CSFV (13). Interestingly, the clinical symptoms of pigs infected with BVDV varied from abortion to inapparent infection (3~5, 14, 15).

Recently, chimeric pestiviruses have been constructed using reverse genetics as candidates for vaccines against classical swine fever (CSF), and these chimeric viruses also are putative marker vaccines for the DIVA (differentiating

Table 1. The properties of five BVDV isolates used in animal experiments

BVD isolate	Origin	CPE (dpi[a])	Genotype[b]
KD26-1	Diarrhea	3~4	1
32669	Persistent infection	4~5	1
95002	Respiratory infection	6~7	2
95589	Diarrhea	4~5	1
32527	Diarrhea	4~5	1

[a] dpi: the number of days after which more than 80% cell death had occurred after inoculation with 0.1 MOI of each virus
[b] Genotype determined by RT-PCR (19)

infected from vaccinated individuals) strategy (16~18). To develop avirulent chimeric pestivirus for the CSFV marker vaccine using BVDV backbone, it is prerequisite to test safety of the candidate BVDV in pigs. Therefore, we decided to evaluate virulence and immunogenicity of the BVDV Korean isolates in pigs and to select one isolate as a candidate for the construction of a marker vaccine against CSF. In the present study, we demonstrated that the five Korean BVDV isolates exhibited weak or no pathogenicity to pigs. We also selected a BVDV isolate to be used for the construction of a chimeric pestivirus.

MATERIALS AND METHODS

Cells and viruses

Madin-Darby bovine kidney (MDBK) cells were propagated in alpha minimal essential medium (alpha MEM; Gibco-BRL, Gaithersburg, MD, USA) supplemented with sodium pyruvate, non-essential amino acids, 5% inactivated horse serum (HS) and antibiotics. Cells were maintained at 37℃ with 5% CO_2.

Four isolates (KD26-1, 32669, 95589, 32527) of BVDV type 1 and one isolate (95002) of BVDV type 2 were prepared for inoculation of pigs. Isolates of BVDV were recovered from cattle with diarrhea or respiratory symptoms in Korea between 1994 and 1995. Details of each isolate are presented in Table 1. For infection of MDBK cells, virus, diluted in alpha MEM, was adsorbed for 1 h at 37℃, and then the inoculum was removed and replaced with fresh alpha MEM-HS. Cultures were incubated at 37℃ for

3 to 6 days until a cytopathic effect (CPE) was observed. Virus stocks were prepared by three freeze-thaw cycles of infected cells in culture medium and clarified by centrifugation at 1000 × g for 10 min.

Animal experiments

Two sets of experiments in swine were performed in order to analyze the five isolates' characteristics of infection. In the first experiment, viral pathogenicity was examined using 40 100-day-old pigs. Pigs were separated into five groups corresponding to the five different virus isolates; each group consisted of six inoculated pigs and two sentinels. Four days before inoculation, blood was collected to confirm that every pig was negative for BVDV specific antigen and antibody. The titers of KD26-1, 32669, 95002, 95589, and 32527 inoculants were $10^{4.8}$, $10^{5.8}$, $10^{6.0}$, $10^{5.5}$, and $10^{5.5}$ $TCID_{50}$/ml, respectively. Pigs were inoculated intranasally with 1 ml of inoculants. Heparinized blood samples and nasal swabs were collected at 0, 1, 2, 3, 4, 5, 7, 9 and 11 days post-inoculation (PI) to check for BVDV antigen by RT-PCR. Additionally, a white blood cell count was performed. Randomly selected pigs were euthanized at 7, 9 and 10 days PI for necropsy to check for gross lesions while tissues from tonsil, bronchial lymph node, lung, spleen, mesenteric lymph node, and ileum were collected for histopathology and to probe for the presence of BVDV antigen by RT-PCR.

In the second experiment, fifteen 40-day-old pigs were used to re-examine the virulence of the KD26-1 isolate. Pigs were separated into two groups. Each of 10 pigs was intramuscularly inoculated with $10^{7.5}$ $TCID_{50}$ of KD26-1 while five pigs as sentinels were housed together with inoculated pigs. Blood samples and nasal swabs were collected at 0, 3, 7, 14, 21 and 28 days PI. Two inoculated pigs and one sentinel were randomly selected and euthanized for necropsy at 3, 7, 14, 21 and 28 days PI. After necropsy, tissues from tonsil, lung, heart, spleen, liver, kidney, mesenteric lymph node, ileum, caecum, inguinal lymph node, and brain were collected and examined for BVDV antigen by RT-PCR and for any gross and histopathological lesions that might have resulted from BVDV infection.

RNA extraction and RT-PCR

RNA was extracted from 300 μl of virus suspension, heparinized blood and nasal swabs using RNeasy Miniprep kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For RNA extraction from tissues, tissue samples were washed three times with autoclaved phosphate-buffered saline (PBS) and 1 g of tissue was removed and ground with sea-sand in a mortar. Ground tissue was mixed with 9 ml of alpha-MEM containing antibiotics. After centrifugation at $1,000 \times g$ for 10 min, 300 μl aliquots of supernatant were used for RNA extraction. RNA was eluted in 50 μl of RNase-free water and stored at -70℃ until use.

Established methods described by Gilbert et al. (19) were slightly modified to carry out cDNA synthesis and RT-PCR for molecular detection and genotyping. Briefly, first-strand cDNA was synthesized using the One Step RT-PCR kit (Qiagen) in a volume of 25 μl containing 5 μl of template RNA, 5 μl of 5 $\times$ RT-PCR buffer, 1 μl of dNTPs (10 mM each), 1 μl of enzyme mix, 0.5 μl of forward and reverse primers (5 pmol each) and 12 μl of RNase-free water. The reaction mixture was incubated at 42℃ for 30 min and then subjected to the following cycles: 94℃ for 15 min followed by 25 cycles at 94℃ for 20 s, 50℃ for 30 s and 72℃ for 30 s. A single 5 min extension step at 72℃ capped the amplification process. For nested PCR, a Qiagen Taq PCR Master Mix Kit was used according to the manufacturer's instructions. The PCR was carried out in 25 μl volumes containing 1 μl of cDNA, 12.5 μl of Taq PCR master mix, 1 μl of nested forward and reverse primers (5 pmol each), and 10 μl of RNase-free water, and the reaction mixture was subjected to the following cycles: 97℃ for 15 min followed by 30 cycles at 94℃ for 20 s, 50℃ for 30 s and 72℃ for 30 s. A single 5 min extension step at 72℃ was again used to cap the amplification process. The PCR products were analyzed by electrophoresis on a 1% agarose gel.

Virus neutralization test

To detect BVDV-specific neutralizing antibodies, a virus neutralization test (VNT) was carried out according to the standard manual of the OIE (20) with flat-bottomed 96-well microtiter plates and MDBK cells. The sera were inactivated for 30 min at 56℃. Briefly, 50 μl of porcine serum was serially diluted two-fold in 50 μl of alpha MEM. A cytopathogenic BVDV (KD26-1 isolate) suspension (200 $TCID_{50}$/50 μl) was added to the wells and mixed on a microplate shaker for 20 s. The plates were then incubated at 37℃ for 1 h and 50 μl of growth medium containing 2 $\times$ 10^5 cells/ml was added to each well. The plates were then incubated for 3 to 4 days in an incubator containing 5% CO_2. The cells were examined for CPE under low-magnification microscopy and translated to a virus neutralization (VN) titer.

RESULTS

Virus characteristics

Five BVDV isolates were categorized into two genetic types using the PCR method suggested by Gilbert et al. (19). Among the five, only isolate 95002 was confirmed by PCR to be type 2. Genetic differences observed for 32669, 95002 and KD26-1 are in agreement with prior sequencing data (21). BVDV type 1 isolates, which were of the CP type, caused more than 80% cell death in MDBK cultures within 4 days after cultivation. The CP biotype of 95002 was not confirmed until 4 days after cultivation. The titer of each isolate did not rise with serial passages, but rather fluctuated up and down.

Pathogenicity of five BVDV isolates in 100-day-old pigs

No significant difference between inoculated pigs and sentinels was found with respect to the leukocyte count after virus inoculation. Most counts fell within normal ranges (Fig. 1). No leukopenia occurred in any of the inoculated or contact pigs, nor were any clinical signs of pathogenic pestivirus observed in this animal experiment. And any gross pathological changes and histopathological findings were not found in euthanized pigs.

Initial virus detection in nasal swabs varied from one

day to three days PI (Table 2) while viral antigens were detected in RNA purified from blood samples as of three days PI (Table 3). In the inoculated pigs, antigen was detectable in nasal swabs and blood samples up to seven days PI. In the sentinel pigs, viral antigen was detected initially from nasal swabs at two days PI. Even though BVD antigens were detected in nasal swabs of inoculated pigs and sentinels in the groups inoculated with isolate KD26-1, 95589, and 32527, the positive patterns of blood

Figure 1. Leukocyte counts (10^3 cells/mm^3) during the course of intranasal infection in the first animal study. Mean leukocyte counts in pigs inoculated with isolates KD26-1, 32669, 95589, 32527 and 95002 and those of contact pigs are given. Standard deviations are shown as error bars.

samples were not consistent with those of nasal swabs. In the group inoculated with KD26-1, BVDV antigens were detected in inoculated pigs and a sentinel. But in the group inoculated with isolate 95589, positive results were shown only in inoculated pigs, and in the group inoculated with isolate 32527, those were shown only in a sentinel.

Though no symptom or clinical sign was apparent in any animal, including sentinels, BVDV antigens were detected in the organs of all inoculated groups except those inoculated with isolate 95002 (Table 4). In the case of the group inoculated with isolate 32669, BVDV antigens were detected in organs of four inoculated pigs and a sentinel. In the group inoculated with KD26-1, viral antigens were detected from tonsil, bronchial lymph node, and lung of inoculated pigs at nine and eleven days PI. And in the group inoculated with isolate 95589 and 32527, viral antigen were detected from tonsil of inoculated pigs. However, spleens, mesenteric lymph nodes, and ileums collected from all groups were negative by RT-PCR. In summary, pigs inoculated with BVDV type 1 were 42%, 38%, and 33% viral antigen positive in nasal, blood and tissue samples, respectively, and pigs inoculated with BVDV type 2 exhibited 67%, 17%, and 0% viral antigen positive rates in nasal, blood and tissues, respectively (Table 5).
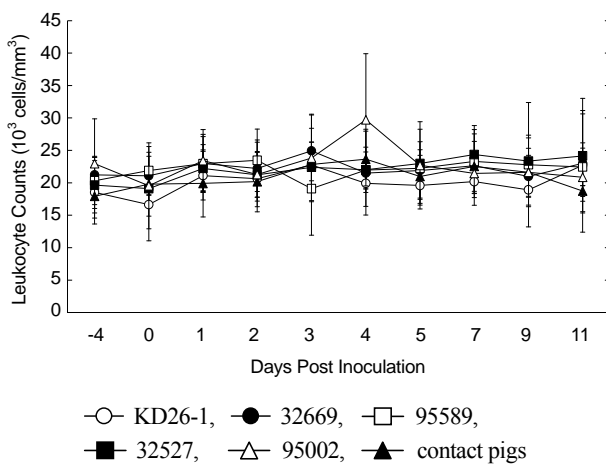
Table 2. RT-PCR results for detection of viral antigen from nasal swabs of five pig groups during the course of intranasal infection

Group	Inoculated virus	Days post inoculation									
		-4	0	1	2	3	4	5	7	9	11
I	KD26-1	0/6[a]	0/6	0/6	**2/6**	0/6	**1/6**	0/6	**2/6**	0/4	0/2
	Contact	0/2	0/2	0/2	0/2	0/2	0/2	**1/2**	**1/2**	0/1	**1/1**
II	32669	0/6	0/6	**1/6**	**1/6**	0/6	0/6	0/6	0/6	0/4	0/2
	Contact	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/1	0/1
III	95002	0/6	0/6	**2/6**	0/6	0/6	**1/6**	**1/6**	**2/6**	0/4	0/2
	Contact	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/1	0/1
IV	95589	0/6	0/6	0/6	0/6	**1/6**	0/6	**1/6**	**1/6**	0/4	0/2
	Contact	0/2	0/2	0/2	**1/2**	**1/2**	0/2	**1/2**	0/2	0/1	0/1
V	32527	0/6	0/6	0/6	0/6	**2/6**	**1/6**	**1/6**	0/6	0/4	0/2
	Contact	0/2	0/2	0/2	0/2	0/2	0/2	**1/2**	**1/2**	**1/1**	0/1

[a] Number of positive pigs / Total number of pigs

Table 3. RT-PCR results for detection of viral antigen from blood samples of five pig groups during the course of intranasal infection

Group	Inoculated virus	Days post inoculation									
		-4	0	1	2	3	4	5	7	9	11
I	KD26-1	0/6[a]	0/6	0/6	0/6	**1/6**	**1/6**	**1/6**	0/6	0/4	0/2
	Contact	0/2	0/2	0/2	0/2	0/2	0/2	**1/2**	0/2	0/1	0/1
II	32669	0/6	0/6	0/6	0/6	0/6	**1/6**	**1/6**	**1/6**	0/4	0/2
	Contact	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/1	0/1
III	95002	0/6	0/6	0/6	0/6	0/6	0/6	**1/6**	0/6	0/4	0/2
	Contact	0/2	0/2	0/2	0/2	0/2	**1/2**	0/2	0/2	0/1	0/1
IV	95589	0/6	0/6	0/6	0/6	0/6	**2/6**	**2/6**	0/6	0/4	0/2
	Contact	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/1	0/1
V	32527	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/4	0/2
	Contact	0/2	0/2	0/2	0/2	0/2	0/2	0/2	**1/2**	0/1	0/1

[a] Number of positive pigs / Total number of pigs

Table 4. RT-PCR detection of viral antigen from tissues samples of five pig groups at 7, 9, and 11 days PI

Group	Inoculated virus	7 dpi[a]	9 dpi	11 dpi
I	KD26-1	–[b]	T[c], B[d], L[e]	T, B, L
	Contact	–	NP[f]	–
II	32669	T, 2B[g], L	L	L
	Contact	T	NP	–
III	95002	–	–	–
	Contact	–	NP	–
IV	95589	–	T	–
	Contact	–	NP	–
V	32527	–	–	T
	Contact	–	NP	–

[a] dpi: days post inoculation, [b] –: negative in tissues, [c] T: positive in tonsil, [d] B: positive in bronchial lymph node, [e] L: positive in lung, [f] NP: not performed, [g] 2B: Two pigs were positive in bronchial lymph node.

Table 5. Summary of viral antigen positive pigs (%) in thirty 100-day-old pigs inoculated intranasally with BVDV type 1 and 2

Virus type	Antigen positive samples			No. of pigs tested
	Nasal swabs	Blood	Tissues	
Type 1	10 (42)	9 (38)	8 (33)	24 (100)
Type 2	4 (67)	1 (17)	0 (0)	6 (100)

Animal experiment with KD26-1 isolate in 40-day-old piglets

The second animal experiment with KD26-1 also failed to reveal any indication of leukopenia. In the course of infection, leukocyte counts were scattered within normal ranges with one exception; the pig among contact pigs presented a leukocyte concentration of 7.4×10^4 cells

/mm^3 at 14 days PI and was diagnosed as Glässer's disease infection by clinical symptoms (Fig. 2). No clinical signs associated with CSFV infection were observed during the course of infection.

According to serological data for the second experiment (Fig. 3), the BVDV specific antibody was not detected until seven days PI. The VN titer ranged from 1:4 to 1:16 at fourteen days PI. By twenty-eight days PI, it had reached to 1:128 and 1:256.

Viral antigen was initially detected in organs at three days PI (Table 6). The organs that were positive for BVDV by RT-PCR were tonsil, spleen, inguinal lymph node and brain. The antigen was detected up to fourteen days PI.

DISCUSSION
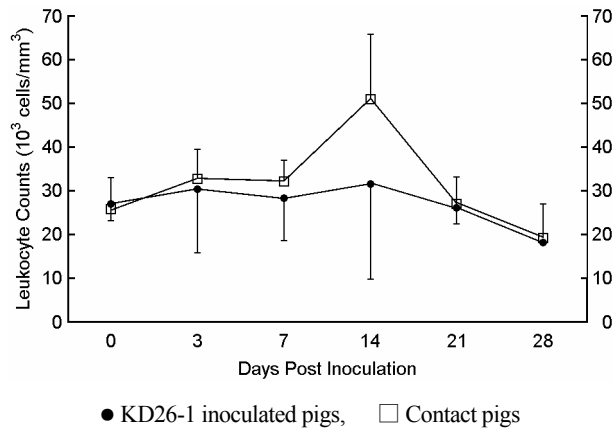
This study was conducted in order to select a BVDV

Figure 2. Leukocyte counts (10^3/mm^3) of pigs after intramuscular inoculation with KD26-1 ($10^{7.5}$ TCID$_{50}$/head). Mean leukocyte counts in pigs inoculated with isolates KD26-1 and those of contact pigs are given. Standard deviations are shown as error bars.
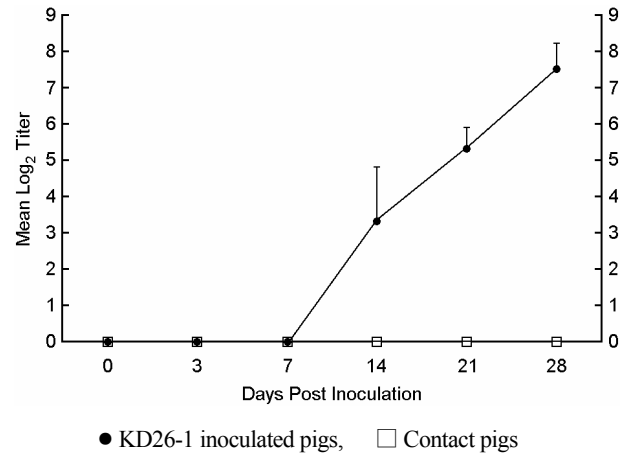

Figure 3. Virus neutralization titers of sera from pigs after intramuscular inoculation with KD26-1 ($10^{7.5}$ TCID$_{50}$/head). Mean virus neutralization titers in pigs inoculated with isolates KD26-1 and those of contact pigs are given. Standard deviations are shown as error bars.

Table 6. RT-PCR results for detection of viral antigen from organs of euthanized pigs after intramuscular inoculation with KD26-1 ($10^{7.5}$ TCID$_{50}$/head)

Organ	KD26-1 inoculated pigs					Contact pigs				
	3 d[a]	7 d	14 d	21 d	28 d	3 d	7 d	14 d	21 d	28 d
Tonsil	**1/2**[b]	**1/2**	**1/2**	0/2	0/2	0/1	0/1	0/1	0/1	0/1
Lung	0/2	0/2	0/2	0/2	0/2	0/1	0/1	0/1	0/1	0/1
Heart	0/2	0/2	0/2	0/2	0/2	0/1	0/1	0/1	0/1	0/1
Spleen	0/2	**1/2**	0/2	0/2	0/2	0/1	0/1	0/1	0/1	0/1
Liver	0/2	0/2	0/2	0/2	0/2	0/1	0/1	0/1	0/1	0/1
Kidney	0/2	0/2	0/2	0/2	0/2	0/1	0/1	0/1	0/1	0/1
Mesenteric LN	0/2	0/2	0/2	0/2	0/2	0/1	0/1	0/1	0/1	0/1
Ileum	0/2	0/2	0/2	0/2	0/2	0/1	0/1	0/1	0/1	0/1
Caecum	0/2	0/2	0/2	0/2	0/2	0/1	0/1	0/1	0/1	0/1
Inguinal LN	0/2	0/2	**1/2**	0/2	0/2	0/1	0/1	0/1	0/1	0/1
Brain	**1/2**	0/2	0/2	0/2	0/2	0/1	0/1	0/1	0/1	0/1

[a] d: days post-inoculation, [b] Number of pigs positive to viral antigen / Number of pigs tested

strain that could be used for the construction of recombinant pestiviruses. At first, it was essential to remove possibly pathogenic strains among our five isolates. Clinical symptoms and gross and histopathological changes were probed because CSF-like pathogenic BVDV isolates have been reported (4, 5, 15), although BVDV normally causes asymptomatic infection in pigs (2, 3, 13, 14). Fortunately, no apparent clinical sign of BVDV infection was presented

during the course of infection, nor was any histopathological change detected.

Next, we examined the infection dynamics of a BVDV viral vector candidate in pigs. It was agreed that a suitable viral vector for construction of a BVDV/CSFV chimeric vaccine should infect swine but disappear from tissues as soon as possible, and it should lack the ability to transmit the virus between pigs. According to the antigen positive

rate of nasal swabs, blood samples, and tissues in the first intranasal pig inoculation experiment, it seemed that five Korean BVDV isolates did not infect pigs effectively. However, the intranasal route of infection to pigs might cause loss of significant amount of inoculants. And, due to the host specificity and the low titer of inoculants, BVDV in pigs might not last long enough to propagate over the detection limit of RT-PCR. In the case of infection with isolate KD26-1, all inoculated pigs in the first animal experiment were not positive by RT-PCR, but VN results in the second animal experiment indicated that serological response to BVDV arouse effectively in pigs. Therefore, the RT-PCR results of the first animal experiment may mean that most of the BVDV isolates were weakly infectious to pigs by intranasal inoculation. Most isolates exhibited similar dynamics during the course of infection except for the 32669 and 95002 isolates. We considered the 32669 isolate to be least desirable among the five isolates since it might be more infectious in pigs than the others, based on antigen detection by the RT-PCR from tissues. In contrast, the 95002 isolate, which is a BVDV type 2, seemed to be too non-infectious to be a vector. Furthermore, due to its slow and ambiguous CPE in cell culture and the dearth of prevalence data regarding BVDV type 2 infection in Korea, it was inappropriate to develop 95002 into a viral vector for CSF control. Among the remaining three isolates, KD26-1 was chosen as a candidate viral vector because of its fast growth rate and quick CPE in cell culture. To ease any concern of pathogenicity in younger pigs, and to obtain serological data after inoculation, we conducted the second inoculation experiment with KD26-1 in piglets.

Characterization of BVDV naturally isolated from pigs and experimental BVDV infection of pigs had been conducted for decades (2~5, 11, 13~15, 22). Persistent virus shedding was demonstrated in congenitally infected pigs that were experimentally induced with ruminant pestiviruses (22). But presently it is accepted that BVDV infection in pig is self-limiting (13). In our first animal experiment, the five BVDV isolates seemed to be actively transmitted between pigs. However, the transmission between pigs could be the result of contamination from remaining inoculant, which had been administered intranasally. This scenario is supported by the fact that antigen was detected one day PI in nasal swabs of inoculated pigs from groups 32669 and 95002 and two days PI in the KD26-1 group, while a nasal swab from contact pigs in the 95589 group were positive two days PI. Yet, the second animal experiment, in which inoculant was administered intramuscularly, failed to demonstrate any cross infection of KD26-1 between pigs despite inoculating with a 500-fold higher titer of KD26-1 than in the first experiment. Therefore, limited transmission of BVDV was demonstrated among pigs with respect to infection with the KD26-1 isolate. However, when pigs are infected with immunosuppressive diseases such as porcine reproductive and respiratory syndrome (PRRS) or post-weaning multisystemic wasting syndrome (PMWS), the possibility of its transmission between pigs remains a question for further study. Interestingly, the route of infection could explain the variable RT-PCR results observed for tissue samples in both experiments. We attempted to use RT-PCR to detect viral antigen from tonsil, bronchial lymph node, lung, spleen, mesenteric lymph node and ileum of all euthanized pigs in the first experiment; however, viral antigen was only detected in tonsil, bronchial lymph node and lung, even though lung tissue from the second experiment failed to reveal viral antigen.

As BVDV is very closely related to CSFV in serology, fears of cross reaction between these often lead to multiple differential diagnoses in order to avoid errors (1, 2, 10, 11, 13). In the second experiment, VN antibodies to BVDV at 28 days PI ranged from 1:128 to 1:256, which might explain elimination of the virus from piglets after only 14 days PI; the VN titer to CSFV was negative during the course of the second experiment. Thus, we can state that serological cross reaction between BVDV and CSFV was not induced by KD26-1 up to 28 days PI.

In the present study, we inoculated pigs with BVDV in order to select a viral vector for development of a live marker vaccine. Though BVDV could be a candidate vaccine to protect pigs from CSFV infection, the main concern of BVDV infection in swine is interference with serological surveillance and the masking of virulent CSFV

by inappropriate and insufficient antibody levels (12, 13). Under mandatory vaccination for CSFV, like that of Korea, DIVA strategy is necessary to differentiate antibodies against field virulent CSFV from those arising from vaccine virus since they cannot be differentiated using conventional serological techniques (23). However, as a part of efforts to prevent any possible virulent factor originating from a DIVA live vaccine, it is also necessary to prove the safety of the original backbone viruses in the target host. We have demonstrated the safety of isolate KD26-1 in pigs by presenting data from the two sets of animal experiments described herein.

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