Application of Restriction Fragment Length Polymorphism Analysis to Simple and Rapid Genotyping of Bovine Viral Diarrhea Virus Strains Isolated in Japan

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ABSTRACT. The E2 regions of 177 bovine viral diarrhea virus (BVDV) strains isolated in Japan between 1957 and 2006 were analyzed for genotyping. The strains were classified into 8 genotypes (1a, 1b, 1c, 1d, 1e, 1f, So and 2a) based on the phylogenetic analysis. The restriction fragment length polymorphism (RFLP) analysis of the RT-PCR products using 6 selected enzymes (*Apo* I, *Mly* I, *BstAP* I, *Pvu* II, *Eae* I, *EcoR* V) disclosed the cutting patterns classified into 11 groups (I–XI), each of that consisted of strains belonging to a single genotype. Namely, groups-I and -II were composed by genotype-1a strains, groups-III and -IV by 1b strains, and groups-V and -VI by 1c strains. Other groups-VII, -VIII, -IX, -X and -XI comprised genotypes-1d, -1e, -1f, -So and -2a strains, respectively. The results suggest that the RFLP analysis can simply and rapidly differentiate the 8 genotypes of BVDV strains.

KEY WORDS: bovine viral diarrhea virus, genotyping, restriction fragment length polymorphism analysis.

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For efficient detection of herds including cattle persistently infected with bovine viral diarrhea virus (BVDV), the screening utilizing serum neutralizing tests, so-called spot tests, have been applied in several countries including Japan, Denmark and U.S.A. [9–11, 17, 20, 21]. The test is most exactly performed when the used indicator virus is antigenically similar to the strain prevalent in the population to be evaluated [17, 20]. And the antigenicities are closely related to the genotypes [8, 16].

The phylogenetic analysis is the most reliable for genotyping but requires a great deal of labor and time as well as preparation of expensive instruments such as a sequencer. On the other hand, several PCR-based assays are used for the simple and rapid genotyping [2, 6, 7, 12, 13, 18, 23], although the range of genotypes classified by the assays is limited to only a few kinds. This report describes the results of the restriction fragment length polymorphism (RFLP) analysis applied to differentiate simply and rapidly the genotypes of BVDV strains prevalent in Japan.

A total of 177 BVDV strains were isolated from 12 Prefectures in Japan during the period from 1957 to 2006, 88 strains from Iwate Prefecture, 41 from Ishikawa Prefecture and the remaining 48 from 10 other Prefectures. Forty-one of 177 strains had been previously classified into genotypes-1a, -1b, -1c, -1e, -1f, -So and -2a based on the analysis of E2 regions [8, 15]. The 175 strains were isolated from diseased cattle and aborted fetuses. The 2 others were recovered from the imported fetal calf sera (FCS). The viruses were cloned by the limiting dilution method and propagated in bovine fetal muscular cells cultivated using the culture medium containing 5% horse serum. The RNA of the

cloned virus was extracted from the supernatant of the infected cells using the TRIzol-LS reagent (Invitrogen, U.S.A.) according to the manufacturer's instruction.

The sense primer 42F (5'-ATG GRC CRG CYT TCC ARA TGG T-3', position in the BVDV SD-1 strain [3]: 2,826–2,847) and the antisense primer 6R (5'-TAG TTG AAG GTG CAT GCC GT-3', position in the strain: 3,357–3,338) were prepared for the amplification of the partial-length E2 genes. The former primer was newly designed in this study and the latter was described previously [22]. The expected products were 532 bp in size. RT-PCR was conducted using SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen, U.S.A.) in a total volume of 50 μ l containing 0.4 μ M of each primer and 1 μ l of the extracted RNA. And the reaction mixtures were heated in a thermal cycler as follows: after cDNA synthesis at 50°C for 30 min and pre-denaturation at 94°C for 2 min, 35 amplification cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min were performed with a final extension step at 72°C for 10 min. Subsequently to the reaction, 5 μ l of each product was electrophoresed on a 2% agarose gel followed by staining with ethidium bromide.

The RT-PCR products were purified by Montage-PCR (Millipore, U.S.A.) and directly sequenced using the same primers and BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, U.S.A.) on Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, U.S.A.). The determined sequences were aligned by the CLUSTAL X method [26] and the phylogenetic tree was constructed using the neighbor-joining method [19] along with the corresponding sequences of the following 18 reference strains: Oregon C24V (accession No. AF091605), Singer (L12455), NADL (M31182), FB1.91/1 (L38697), SD-1 (M96751), CP7 (U63479), New York-1 (AY027671), Osloss

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(M96687), Glen Innes (AF049226), Deer-NZ1 (AF144614), Bega (AF049221), SH9 (AF144616), 721 (AF144609), Deer-GB1 (AF144615), 11468 (AY734488), Giessen-1 (AF104030), 890 (U18059) and Gi-6 (AF144612). The reliability of the tree was evaluated by bootstrap analysis carried out on 1,000 replicates [5].

The cutting sites by the restriction endonucleases were investigated on the sequences of 177 strains using the Map-Draw software (DNASTAR, U.S.A.), and 6 enzymes including *Apo* I, *Mly* I, *BstAP* I, *Pvu* II, *Ear* I and *EcoR* V (New England BioLabs, U.S.A.) were selected as candidates for genotyping. On RFLP analysis of 177 strains, 5 μ l of RT-PCR products were added to 15 μ l of the mixtures containing 10 units of an enzyme and reacted under the conditions recommended in the instructions. The digested fragments were visualized as mentioned above.

RT-PCR using 42F and 6R primers successfully amplified the products of the expected sizes from all 177 examined strains. The determined nucleotide sequences revealed that all RT-PCR products were 532 bp in size without deletion or insertion. On the phylogenetic tree based on the 490 nucleotides excluding positions annealed by the primers, 63, 69 and 18 of 175 strains were classified into genotypes-1a, -1b and -1c, and 4, 2, 2 and 17 strains into -1e, -1f, -So and -2a, respectively. The 2 others from the imported FCS belonged to the genotype-1d. The bootstrap values of the clusters constructing each genotype were more than 94%. The present reanalysis of 41 strains disclosed the identical genotypes with the previous results [8, 15].

The electrophoretic patterns observed on RFLP analysis were divided into 11 groups (I-XI) as shown in Table 1. Each group consisted of strains belonging to a single genotype. Namely, groups-I and -II were composed by genotype-1a strains, groups-III and -IV by 1b strains, and

groups-V and -VI by 1c strains. Other groups-VII, -VIII, -IX, -X and -XI comprised genotypes-1d, -1e, -1f, -So and -2a strains, respectively.

The RT-PCR products obtained from 37 (group-I) of 63 genotype-1a strains were digested into 265 and 267 bp in size by *Apo* I. *Mly* I also digested the products into 146 and 386 bp, with the exception that one strain (Nose) showed the digestion pattern of 106, 146 and 280 bp. None of the 4 other enzymes digested the products of this group. The 26 others (group-II) of genotype-1a strains showed the identical patterns with group-I strains by *Apo* I digestion. None of the 5 other enzymes digested the products, with the exception that one strain (IW20/97/NCP) were cut into 190 and 342 bp by *Mly* I and 2 strains (IW59/05/NCP, IS21NCP/00) into 174 and 358 bp by *BstAP* I.

The products of 69 genotype-1b strains were digested with 2 main patterns by *Mly* I and not by the 5 other enzymes. Namely, 22 strains (group-III) were cut into 252 and 280 bp, and the 47 others (group-IV) into 95, 185 and 252 bp with the exception of 2 strains (IS3NCP/95, IS6NCP/95). The latter 2 strains showed the digestion patterns of 95, 118, 134 and 185 bp.

Fourteen (group-V) of 18 genotype-1c strains were digested into 256 and 276 bp by *BstAP* I and not by the 5 other enzymes with the exception of the following 3 strains. Two strains (IW1/87/NCP, TC TA142/01) were cut into 265 and 267 bp by *Apo* I and 3 strains (IW1/87/NCP, IW4/89/NCP, TC TA142/01) into 252 and 280 bp by *Mly* I. The 4 other genotype-1c strains (group-VI) were digested into 52 and 480 bp by *Pvu* II. Three strains (IW56/05/CP, IW56/05/NCP, IW63/06/NCP) were also cut into 256 and 276 bp by *BstAP* I.

Two genotype-1d strains (group-VII) were digested into 262 and 270 bp by *Ear* I. One strain (OK1(CA)NCP/03)

Table 1. Grouping of 177 examined BVDV strains based on the RFLP patterns

Group	Genotype	No. of strains	The size (bp) of fragments generated by					
			<i>Apo</i> I	<i>Mly</i> I	<i>BstAP</i> I	<i>Pvu</i> II	<i>Ear</i> I	<i>EcoR</i> V
I	1a	37	265, 267	146, 386 ^{a)}	nd	nd	nd	nd
II	1a	26	265, 267	nd ^{b)}	nd ^{c)}	nd	nd	nd
III	1b	22	nd	252, 280	nd	nd	nd	nd
IV	1b	47	nd	95, 185, 252 ^{d)}	nd	nd	nd	nd
V	1c	14	nd ^{e)}	nd ^{f)}	256, 276	nd	nd	nd
VI	1c	4	nd	nd	256, 276 ^{g)}	52, 480	nd	nd
VII	1d	2	nd	nd	nd ^{h)}	nd	262, 270	nd
VIII	1e	4	265, 267	nd	nd	218, 314	nd	nd
IX	1f	2	nd	nd	nd	nd	nd	nd
X	So	2	nd	252, 280	256, 276	175, 357	nd	nd
XI	2a	17	nd	nd	nd	nd	nd	167, 365

nd: Not digested.

a) Except Nose digested into 106, 146 and 280 bp for addition of one cutting site.

b) Except IW20/97/NCP digested into 190 and 342 bp.

c) Except IW59/05/NCP and IS21NCP/00 digested into 174 and 358 bp.

d) Except IS3NCP/95 and IS6NCP/95 digested into 95, 118, 134 and 185 bp.

e) Except IW1/87/NCP and TC TA142/01 digested into 265 and 267 bp.

f) Except IW1/87/NCP, IW4/89/NCP and TC TA142/01 digested into 252 and 280 bp.

g) Except Okazaki/01/04 not digested for loss of one cutting site.

h) Except OK1(CA)NCP/03 digested into 174 and 358 bp.

was also cut into 174 and 358 bp by *BstAP* I. None of the 4 other enzymes digested the products. Four genotype-1e strains (group-VIII) disclosed the patterns of 265 and 267 bp by *Apo* I as well as 218 and 314 bp by *Pvu* II. Two genotype-1f strains (group-IX) had no cutting sites of the 6 enzymes. Two genotype-So strains (group-X) disclosed the patterns of 252 and 280 bp by *Mly* I, 256 and 276 bp by *BstAP* I and 175 and 357 bp by *Pvu* II. Seventeen genotype-2a strains (group-XI) were cut into 167 and 365 bp only by *EcoR* V.

Previous phylogenetic analysis of E2 genes has often been accomplished based on the RT-PCR and sequencing using plural primer sets [1, 25], since the regions are considerably variable [3, 4]. In this study the RT-PCR using a single primer set consisting of the newly designed 42F and previously reported 6R [22] successfully amplified the regions of all 177 examined strains, which were subsequently divided into 8 genotypes based on the nucleotide sequences determined by the primer set. On the phylogenetic tree, the clusters constructing each genotype were supported by the bootstrap values of more than 94% and reanalysis of 41 strains disclosed the identical genotypes with previous results [8, 15]. The subjected E2 regions contained the 420 nucleotides that had often been analyzed phylogenetically in Japan [15, 24]. These results suggest that the present primer set could contribute to the phylogenetic analysis of E2 genes.

Based on the phylogenetic analysis, the examined strains were frequently divided into genotypes-1a and -1b, occasionally genotypes-1c and -2a and rarely -1e, -1f and -So, and included 2 genotype-1d strains from the imported FCS. The obtained results appear to suggest that the examined strains could be representative of Japanese isolates from the view of the kinds and frequency of the detected genotypes [8, 14–16, 24].

The RFLP patterns obtained from 177 strains using 6 selected enzymes were divided into 11 groups (I–XI). The identical cutting patterns were found in most strains within each group and the different patterns were observed in a few strains. However, the patterns of the latter minor strains were differentiated from those of the strains within the other groups when analyzed by the present 6 enzymes. Each group consisted of strains belonging to a single genotype, such as genotype-1a strains in groups-I and -II, 1b strains in groups-III and -IV, and 1c strains in groups-V and -VI. The differences in the cutting patterns among the 11 groups were easily visualized by the present electrophoresis. The results suggest that the present RFLP analysis could simply and rapidly differentiate compared with the phylogenetic analysis the 8 genotypes of strains that have so far been isolated in Japan, although no enough strains to evaluate in number were examined on several genotypes.

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