PHYLOGENETIC ANALYSIS OF PESTIVIRUS BASED ON THE 5'-UNTRANSLATED REGION

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Summary. – A phylogenetic tree of pestiviruses constructed by analyzing their 5'-untranslated region (UTR) indicated that the genetic relatedness between border disease virus and hog cholera virus is much closer than that between genotypes of various bovine diarrhoea viruses. This suggests that these viruses are host variants within a single species, which can be distinguished by comparison of secondary structures at three variable loci in the 5'-UTR.

Key words: border disease virus; bovine diarrhoea virus; hog cholera virus; pestivirus

The pestiviruses are small enveloped viruses containing a single-stranded, positive-sense RNA genome of about 12.5 kb in length, which are classified as the *Pestivirus* genus of the *Flaviviridae* family (Wengler *et al.*, 1995). Although the genus *Pestivirus* includes currently three species, border disease virus (BDV) of sheep and goats, bovine diarrhoea virus (formerly bovine viral diarrhoea virus, BVDV) of cattle, and hog cholera virus (HCV) of swine, serological surveys indicate that the host range of pestiviruses includes most even-toed ungulates (Nettleton, 1990). In addition, pestivirus infections of humans have been frequently suspected (Wilks *et al.*, 1989; Yolken *et al.*, 1989), and a pestivirus has been isolated from a patient (Giangaspero *et al.*, 1993). A non-cytopathic pestivirus has been known not only as a veterinary pathogen but also as a common contaminant in animal cell cultures or foetal bovine sera (Bolin *et al.*, 1994).

The pestivirus genome has a relatively long 5'-UTR upstream the polyprotein open reading frame. The nucleotide sequence of the 5'-UTR is well conserved among the members of the *Pestivirus* genus (Qi *et al.*, 1993). In general, the 5'-UTR of positive-sense RNA viruses includes regulatory motifs for RNA transcription or translation, which are usually composed of a combination of primary, secondary and tertiary structures. A prokaryotic-like motif called the box A-box B tandem and assigned in the 5'-UTR of pestivirus genome (Brown *et al.*, 1992) has been proposed to be a potential 18 S ribosomal RNA binding site contributing to the translational strategy such as cap-independent translation initiation mechanism (Le *et al.*, 1995) or internal translation through the internal ribosome entry site (Deng and Brock, 1993). A reverse transcription – polymerase chain reaction (RT-PCR) allows a direct detection of pestivirus RNA in clinical specimens. The RT-PCR products can be subsequently directly sequenced (Hofmann *et al.*, 1994). In this article we searched for consensus structure in the 5'-UTR of genomic RNA of various pestiviruses with the aim to define pestivirus species and/or genotypes.

Nucleotide sequences in the 5'-UTR of pestiviruses BDV, BVDV and HCV were obtained from previous publications of the author's laboratory and from the DNA databases, and were aligned by the method of Higgins *et al.* (1992). The sequence alignment indicated that the pestivirus genome includes at least three variable loci in the 5'-UTR (Fig. 1). A phylogenetic tree of pestiviruses was constructed from the nucleotide sequences in the 5'-UTR by the neighbor-joining method of Saitou and Nei (1987). The phylogenetic tree indicated that the genetic relatedness between BDV and HCV is much closer than that between the genotypes II and Ia or Ib of BVDV (Fig. 2). This may remove the species

Abbreviations: BDV = border disease virus; BVDV = bovine diarrhoea virus; HCV = hog cholera virus; RT-PCR = reverse transcription-polymerase chain reaction; UTR = untranslated region

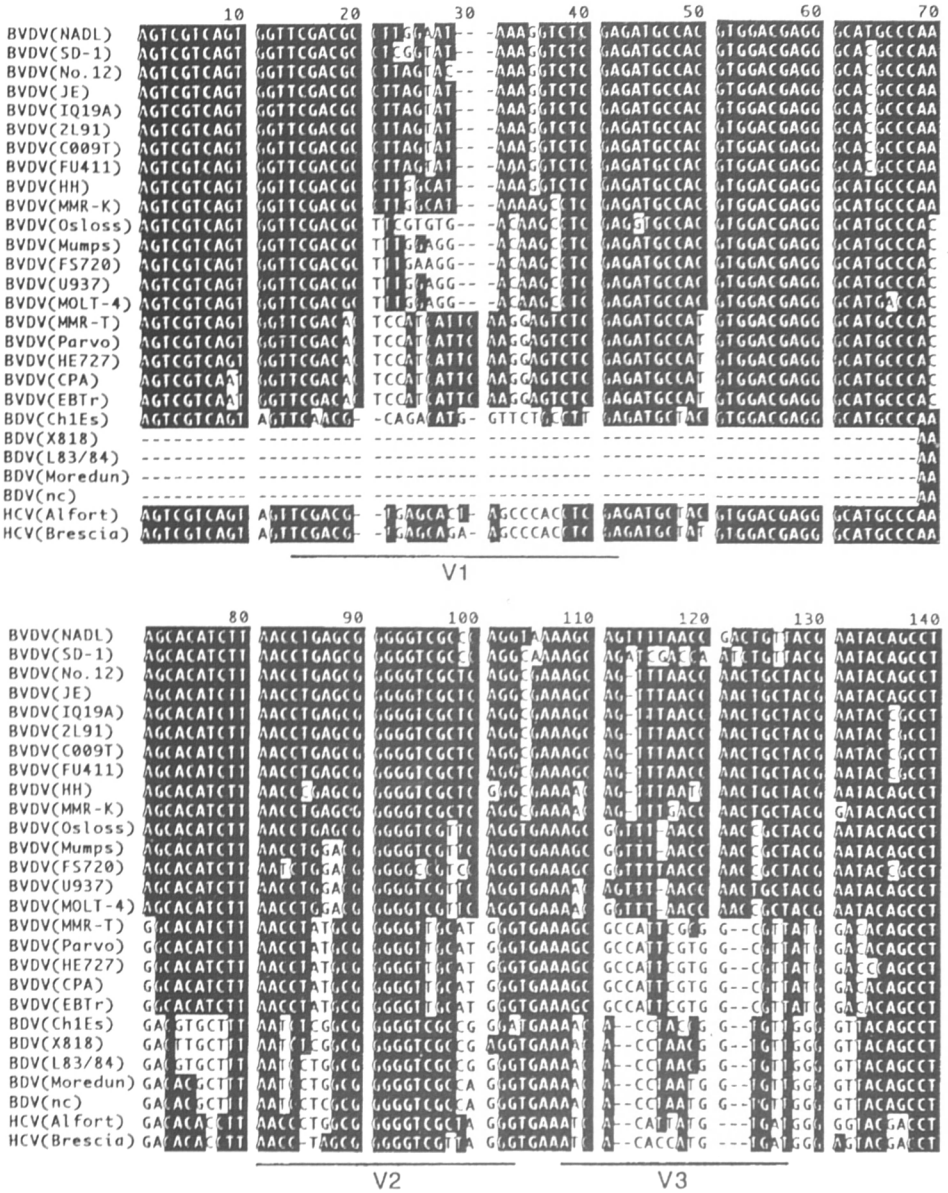

Fig. 1

Nucleotide sequence alignment of the PCR products

The nucleotide numbers are given by the consensus alignment. Nucleotides that are identical in two out of three sequences are shown in white on black. The nucleotide sequences of BVDV strains NADL (Collett *et al.*, 1989), Osloss (De Moerlooze *et al.*, 1993) and SD-1 (Deng and Brock, 1992), of BDV strains L83/84 (Becher *et al.*, 1995), Moredun (Becher *et al.*, 1995), X818 (Becher *et al.*, 1995) and nc (De Moerlooze *et al.*, 1993), and HCV strains Alfort (Meyers *et al.*, 1989) and Brescia (Moormann *et al.*, 1990) were taken from the indicated publications. The nucleotide sequences of other strains were taken from publications by the present author and his colleagues (Harasawa, 1994; Harasawa, 1995; Harasawa and Mizusawa, 1995; Harasawa and Sasaki, 1995; Harasawa and Tomiyama, 1994). Dashes represent space between adjacent nucleotides introduced for maximum alignment. Three variable region loci, V1, V2 and V3, are underlined.

barier between BDV and HCV in terms of nucleotide homology. If not, this may indicate that the genotype II of BVDV is a novel species of the genus *Pestivirus*. Thiel and his coworkers have recognized this new group as **pestivirus type 4** in their phylogenetic tree, and they also have located BDV closer to HCV than to BVDV (Becher *et al.*, 1994; Becher *et al.*, 1995).

They have proposed to designate classical BVDV strains **pestivirus type 1**, HCV strains **pestivirus type 2**, and "true" BDV strains **pestivirus type 3** (Becher *et al.*, 1995). The

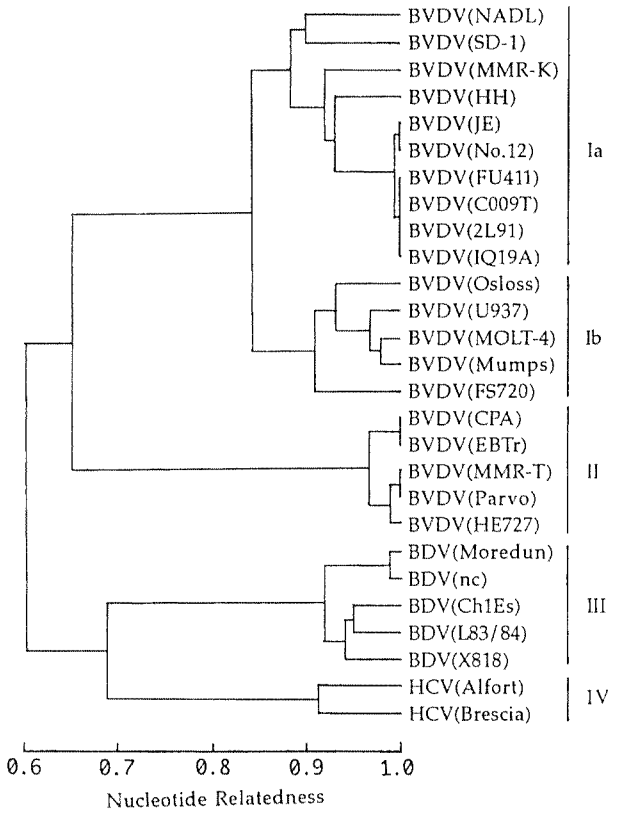

Fig. 2
Dendrogram showing degree of nucleotide sequence relatedness among 27 pestiviruses determined by the neighbor-joining method

BDV strain BD-87 which has been reported to be closer to BVDV than to HCV (Sullivan *et al.*, 1994) does not seem to be a "true" BDV strain because its 5'-UTR was identical to the BVDV group II of Pellerin *et al.* (1994) or to the BVDV genotype II in the present article (Fig. 2).

The secondary structure of the 5'-UTR was predicted according to the algorithm of Zuker and Stiegler (1981) using the DNASIS program package (Hitachi Software Engineering, Co., Yokohama, Japan). The three variable loci V1, V2 and V3 in the 5'-UTR of pestiviruses were capable to form a stable stem-loop structure (Fig. 3). Stem-loop structures in the 5'-UTR presented in this article may serve as a useful reference for the genotype identification of a pestivirus, and may provide an additional procedure for identification and classification of pestivirus isolates.

Typical stem-loop structures at the V1 locus are shown on the top of Fig. 3. Although the loop regions are variable both in size and sequence among the pestivirus species or genotypes, several nucleotides of the loop region are strictly conserved within a species or genotype. The C:C pairing that forms a bulge in the helix region was defined as an

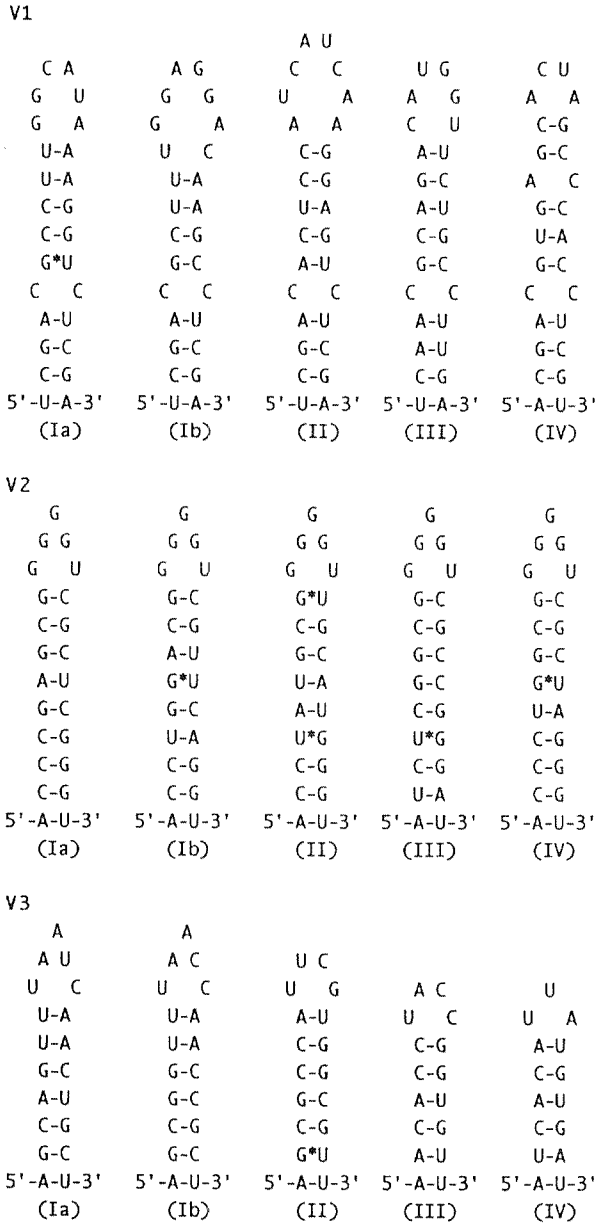

Fig. 3
Typical secondary structures predicted for the variable region loci V1, V2 and V3 in the 5'-UTR of RNA of pestivirus genotypes
The Watson-Crick base pairing is indicated by a dash and the G:U pairing tolerated in secondary structures by asterisk.

obligatory consensus feature which divides the helix into top and bottom helices. Four or five base pairs form the top helix which is mandatory. The bottom helix includes four base pairs. The bottom helix in the genotypes Ia, Ib and II is strictly conserved and is distinct from that of BDV (genotype III) or HCV (genotype IV). Another bulge caused by the A:C pairing is conserved in genotype IV.

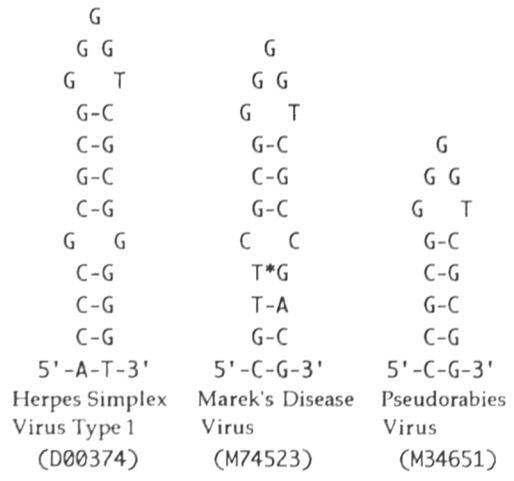

Fig. 4
Secondary structures predicted for the internal long repeats of DNA of three herpesviruses, which are similar to those of the V2 loci of pestiviruses
The Watson-Crick base pairing is indicated by a dash, and the T:G pairing tolerated in secondary structures by asterisk. Accession numbers of the nucleotide sequences are given in parentheses.

Typical stem-loop structures at the V2 locus are shown in the middle of Fig. 3. The sequence 5'-GGGGU-3' of the loop region was defined as a consensus motif in this GC-rich secondary structure among all the members of the genus *Pestivirus*. Nucleotides of the stem region are variable and covariant to support stable secondary structures in different types of pestiviruses. Similar secondary structures with a nucleotide sequence 5'-GGGGT-3' of the loop region were found in the internal long repeats in herpesviruses (Fig. 4). Biological significance of this particular type of structure is currently unknown.

Typical stem-loop structures at the V3 locus are shown in the bottom of Fig. 3. The helix structures were well supported by covariant substitutions, even though nucleotide sequences were variable in the stem and loop regions in different genotypes.

Four independent schemes for the BVDV genotyping have been proposed on the basis of comparison of the 5'-UTR (Becher *et al.*, 1995; Harasawa, 1994; Pellerin *et al.*, 1994; Ridpath *et al.*, 1994). Three of them depend exclusively on the comparison of primary structure of the 5'-UTR (Becher *et al.*, 1995; Pellerin *et al.*, 1994; Ridpath *et al.*, 1994). It is certainly reasonable to compare the nucleotide sequences of the structural genes or translated regions in viruses to determine their genetic relatedness (Hertig *et al.*, 1995). However, in comparing the control regions such as the 5'-UTR it is more meaningful to examine their secondary or tertiary structure. The hypothetical stem-loop structures at the three variable loci in the 5'-UTR were characteristic for each genotype, and were not shared by any of other genotypes. This may represent a key feature for the classification and identification of pestiviruses. We have previously proposed a scheme which consists of three genotypes of BVDV according to the comparison of the secondary structures of their 5'-UTR (Harasawa, 1994). BVDV genotypes I, II and III in that scheme corresponded to the subgroups Ia and Ib and group II, respectively, proposed by Pellerin *et al.* (1994). In the genotype systems of Ridpath *et al.* (1994) and Becher *et al.* (1995), the subgroups Ia and Ib proposed by Pellerin *et al.* (1994) have been united into a single type. In the present article, to avoid confusion of various genotyp nomenclatures, we propose five groups of pestiviruses consisting of genotypes Ia and Ib for BVDV, genotype IV for HCV, genotype III for BDV, and genotype II for the new type of BVDV

Table 1. Nomenclature of pestiviruses

Pestivirus strains*	Pestivirus genotypes			
	Harasawa (this article)	Pellerin *et al.* (1994)	Ridpath *et al.* (1994)	Becher *et al.* (1995)
NADL,SD-1,No.12 Oregon(C24V),VM	Ia	Ia	I	Pestivirus type 1
Osloss,NY-1,Draper Sanders,TGAN	Ib	Ib	I	Pestivirus type 1
890,CD87,EBTr	II (new type)	II	II	Pestivirus type 4
Moredun,L83/84,Ch1Es	III	NA	NA	Pestivirus type 3
Alfort, Brescia	IV	NA	NA	Pestivirus type 2

*Strains NADL (Collett *et al.*, 1989), Osloss (De Moerlooze *et al.*, 1993), SD-1 (Deng and Brock, 1992), No.12 (Harasawa, 1994), Oregon(C24V) (Pellerin *et al.*, 1994; Ridpath *et al.*, 1994), VM (Qi *et al.*, 1993; Ridpath *et al.*, 1994), NY-1 (Pellerin *et al.*, 1994; Ridpath *et al.*, 1994), Draper (Pellerin *et al.*, 1994), Sanders (Qi *et al.*, 1993; Ridpath *et al.*, 1994), TGAN (Qi *et al.*, 1993; Ridpath *et al.*, 1994), 890 (Pellerin *et al.*, 1994; Ridpath *et al.*, 1994), CD87 (Pellerin *et al.*, 1994), EBTr (Harasawa and Mizusawa, 1995), Moredun (Becher *et al.*, 1995), L83/84 (Becher *et al.*, 1995), Ch1Es (Harasawa and Mizusawa, 1995), Alfort (Meyers *et al.*, 1989), and Brescia (Moorman *et al.*, 1990). NA = not applicable.

(Table 1). These groups are defined by comparing the secondary structures of their 5'-UTR (Fig. 3).

Although the pestivirus species are classified predominantly according to the animal host species from which they were isolated, there is an extensive antigenic cross-reactivity among them, and they can cross the host species barrier and infect different animal species within the cloven-footed ungulates (Edwards *et al.,* 1995; Paton *et al.,* 1992; Terpsata and Wensvort, 1988). Such cross-infections may obscure the rationale for the definition of the pestivirus species according to their host. Therefore it is essential to establish a satisfactory criterion for the definition of pestivirus species (Horzinek, 1995). The phylogenetic tree constructed in the present study suggests that BDV, BVDV and HCV are host variants within a single species, though their host specificity is not so determinative.

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