Genotypic Characteristics of *Bovine Viral Diarrhea Virus 2* Strains Isolated in Northern Italy

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ABSTRACT. Two strains of *Bovine viral diarrhea virus 2* (BVDV-2) were isolated from calves in northern Italy. Variations in the 5'-untranslated region (UTR) of the genome were studied by primary structure alignment and neighbor-joining method based phylogenetic tree analyses and by palindromic nucleotide substitutions at the three variable loci in the 5'-UTR. Genetic analysis indicated their appurtenance to genovar BVDV-2a. Nucleotide sequence at the 5'-UTR of strain BS-95-II, one of the Italian isolates from healthy calves, showed 98% homology to that of the Japanese isolate OY89, a cytopathic strain derived from cattle with mucosal disease.

KEY WORDS: *Bovine viral diarrhea virus 2*, genovar, pestivirus

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Bovine viral diarrhea virus 2 (BVDV-2) reported from outbreaks characterized by thrombocytopenia and high mortality of cattle in U.S.A. and Canada [18, 20] as well as demonstrated in a human live virus vaccine in 1994 [13] is an economically important, viral species along with *Bovine viral diarrhea virus 1* (BVDV-1), *Border disease virus* (BDV) and *Classical swine fever virus* (CSFV), of the genus *Pestivirus* within the family *Flaviviridae* [25].

According to the classification proposed by Pellerin *et al.* [18] and further applications [5–8], based on the nucleotide divergence in the 5'-untranslated region (UTR) of genomic RNA, the BVDV-1 can be segregated into at least four genotypes or genovars, BVDV-1a, BVDV-1b, BVDV-1c and BVDV-1d. Genovars BVDV-1a and BVDV-1b represent the majority of classical BVDV strains such as NADL, SD-1, Oregon (C24V), Singer, Osloss and NY1 reference strains. Strain Europa, a non-cytopathic (NCP) BVDV strain isolated from a human buffy coat sample [5, 6] and three bovine field isolates [27] were classified as the genovar BVDV-1c. BVDV-1d includes strains detected as contaminants of biologicals [7]. CSFV strains were divided into at least three genovars [10]. CSFV-1 genovar includes the reference strain Brescia, and CSFV-2 genovar includes the reference strain Alfort. The two Japanese strains Kanagawa/74 and Okinawa/86 were classified in the CSFV-3 genovar. A novel taxon in the genus *Pestivirus* has been suggested for a giraffe strain [11, 25]. BVDV-2 includes the strains 890 and CD87 isolated from first described outbreaks of haemorrhagic syndrome in cattle in U.S.A. and Canada [18, 20]. BVDV-2 strains can be segregated into four genovars, BVDV-2a, BVDV-2b, BVDV-2c and BVDV-2d, by palindromic nucleotide substitutions (PNS) at V1, V2 and V3 variable loci at the 5'-UTR genomic

region and by phylogenetic analyses [4]. Genovars BVDV-2a and BVDV-2c represented only strains isolated from cattle. In genovar BVDV-2b were classified both cattle and sheep isolates. Thus, only strains from sheep were included in genovar BVDV-2d.

BVDV was described for the first time in Italy in 1958 in the province of Matera and short after in northern Italy [19]. Recently, in a retrospective screening, two BVDV-2 strains were isolated in buffy coat samples originated from cattle, from the northern Italian provinces of Brescia and Milan, collected in 1995 and in 1997, respectively [16]. Both originated from healthy calves. The present study focused on the genetic characterization of the 5'-UTR of these two virus isolates.

Primary structures at the 5'-UTR of the two BVDV-2 strains isolated from calves in northern Italy, strain BS-95-II (accession number AJ288903) and strain 11/Mi/97 (accession number AJ293603), have been deposited to the DNA database. Other nucleotide sequences of the pestivirus genome were all obtained from the publications or from the DNA databases, and considered for genetic analysis. The nucleotide sequences of BVDV-2 strains, CD87 [18], 890 [18], SCP [1], BD78 [22], MnFetus [20], MadSpl [20], AzSpl [20], 175375 [26], 173157 [26], 168149 [26], Lees [26], EBTr [12], MMR-T [13], strains 17011 [24], 713 [24], 5521 [24], NY93 [24], 17583 [24], 23025 [24], 7937 [24] and OY89 [17], were used for comparison.

Nucleotide sequences from the 5'-UTR of pestivirus RNA were aligned by the Clustal W [23]. A phylogenetic tree depending on the primary structures of the 5'-UTR among the known pestivirus RNA was constructed by the neighbor-joining method by Saitou and Nei [21]. Secondary structures were predicted according to the algorithm of Zuker and Steigler [28]. Folding energies of the secondary structures were calculated by the method of Freier *et al.* [3]. Genotyping was performed according to the method

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Fig. 1. Nucleotide sequence alignment for the 5'-UTR for sequence comparison of the strains BS-95-II and 11/Mi/97 with 21 published pestivirus sequences of BVDV-2 strains. Nucleotides that are identical in two out of three sequences are shown as inverted characters. The nucleotide sequence numbers are given from a consensus alignment. Dashes represent spacers between adjacent nucleotides introduced for maximum alignment. Three variable regions, V1, V2 and V3, are underlined.

described by Harasawa and Giangaspero [9]. Relevant variations in the three variable loci, V1, V2 and V3, characteristic stem-loop palindromic sequences, at the 5'-UTR were considered for the phylogenetic analysis.

The analyses, based on nucleotide sequence homology and on nucleotide relevant variations in the 5'-UTR, containing characteristic palindromic structures, indicated that the two strains BS-95-II and 11/Mi/97 belonged to the BVDV-2a genovar. Comparison of the 5'-UTR nucleotide sequence in the BS-95-II and 11/Mi/97 strains with those in other 21 published BVDV-2 sequences was performed by the Clustal W alignment program (Fig. 1). A dendrogram

based on primary nucleotide sequence comparison was obtained by the neighbor-joining method (Fig. 2).

The overall nucleotide sequence homology between the BS-95-II and 11/Mi/97 strains was of 93%. The homologies between BS-95-II strain and BVDV-2a, BVDV-2b, BVDV-2c and BVDV-2d strains were of 92 to 98%, respectively. The nucleotide sequence values of the strain BS-95-II against genotypes BVDV-1 were of 69–72%. Similarly, the homologies between the strain BS-95-II and BDV, and between the strain BS-95-II and CSFV were 66–69% and 67–69%, respectively. The homologies between 11/Mi/97 strain and BVDV-2a, BVDV-2b, BVDV-2c and BVDV-2d

Fig. 2. Phylogenetic tree constructed from the 5'-UTR of BVDV-2 strains, segregated into four genovars, BVDV-2a, BVDV-2b, BVDV-2c and BVDV-2d, according to the neighbor-joining method.

strains were of 92 to 97%, respectively. The nucleotide sequence values of the strain 11/Mi/97 against genotypes BVDV-1 were of 68-69%. The homologies between the strain 11/Mi/97 and BDV were of 63-66% and with CSFV of 66-67%. The BS-95-II strain derived from a healthy calf showed 98% homology to the Japanese isolate OY89, a cytopathic strain isolated from blood samples of cattle with

mucosal disease in 1989 [17].

Sequence variation within the 5'-UTR of the BS-95-II and 11/Mi/97 strains, mainly limited to three specific regions, the variable loci V1, V2 and V3, typical palindromic structures, stem-loop shaped, were found by manual search through potential secondary structures, looking for substitutions of one Watson-Crick base pair for another.

Fig. 3. Secondary structures predicted for the three variable regions, V1, V2 and V3, in the 5'-UTR in the two BVDV-2 strains BS-95-II (AJ288903) and 11/Mi/97 (AJ293603) isolated in calves in northern Italy. Watson-Crick base-pairing is indicated by a dash, the G and U pairing tolerated in secondary structures by an asterisk, and inter-changeable base-pairing by a colon.

Pestivirus-characteristic consensus motifs by PNS, at the V1 and V2 regions were identified: the C:C bulge at position 19 from the bottom of the variable locus V1 stem; A-U base-pairing at position 18, C-G base-pairing and G-C base-pairing at positions 12 and 16, respectively; the nucleotide sequence 5'-GGGGU-3' in the V2 loop (exception made for BVDV-2 7937 strain with C instead of U nucleotide) and C-G base-pairing at position 3 from the bottom of the V2 stem. BVDV-2 specific PNS were observable in the three variable loci. Base pairings C-G at position 23 and 24 in the V1 stem, the G and U pairing tolerated in secondary structures at position 9 in the V2 stem and C-G base-pairing at position 5 in the V3 stem. Predicted secondary structure of the 5'-UTR of the BS-95-II and 11/Mi/97 strains are represented in Fig. 3. In strain BS-95-II, the stable stem-loop palindromic structures showed substantial negative free energies: -109.77 KJ/mol for the stem-loop V1, -51.95 KJ/mol for the stem-loop V2 and -27.65 KJ/mol for the stem-loop V3. Simi-

larly, in strain 11/Mi/97, free energy values were: -102.65 KJ/mol for the stem-loop V1, -31.84 KJ/mol for the stem-loop V2 and -47.34 KJ/mol for the stem-loop V3. These negative values in free energies support stable secondary structures predicted for the three variable regions.

The analyses based on primary nucleotide sequence homology and on secondary palindromic sequence structures characteristic to each genovar of the 5'-UTR of pestivirus RNA revealed that the BVDV-2 strains BS-95-II and 11/Mi/97 should be assigned to the genovar BVDV-2a. The results obtained by the evaluation of the sequence divergence at secondary structure level, in the three palindromic V1, V2 and V3 loci in the 5'-UTR were comparable to those obtained by the nucleotide sequence alignment and the phylogenetic tree obtained from comparison of the nucleotide sequences in the 5'-UTR among known pestivirus RNA.

In addition to the haemorrhagic syndrome, the BVDV-2 in cattle was associated with abortion, acute BVD and sub-

clinical persistent infection [24]. In sheep the infection was clinically similar to border disease [1, 22, 26]. Genovars BVDV-2a and BVDV-2b included isolates with low virulence as well as strains with high virulence, but, in genovars BVDV-2c and BVDV-2d were present only high virulent ones.

BVDV-2 strains were also detected as contaminants in biological products and cell cultures [8]. Contamination of cell cultures by virulent strains of BVDV-2 may be due to the use of infected fetal bovine serum as culture medium supplement, and this may represent a source of viral diffusion in cattle population. In this context, a relevant case was recently reported. An outbreak of haemorrhagic syndrome in Dutch cattle was caused by an infectious bovine rhinotracheitis live virus vaccine contaminated with a virulent BVDV-2 strain [2]. Fetal bovine serum was used as medium supplement for the substrate cells used for the production of these vaccines and it was the most probable source of contamination, despite certification of BVDV antibody and antigen absence and irradiation procedures. Occurrence of BVDV iatrogenic infection in animals has been previously reported, in relation with vaccines contaminated by infectious pestivirus [14, 15].

In conclusion, the characterization of the BS-95-II and 11/Mi/97 strains of BVDV-2, based on analyses of primary and secondary structures of the 5'-UTR, indicated the appurtenance to genovar BVDV-2a. The identification of genetic variations based on nucleotide substitutions in the genome of BVDV-2 strains might improve our understanding in terms of evolutionary and epizootiological history, and might provide genetic markers for biological differences.

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