NOTE  Virology

Genetic Variety of Bovine viral diarrhea virus 2 Strains Isolated from Sheep

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ABSTRACT. Bovine viral diarrhea virus 2 (BVDV-2) strains, isolated from sheep showing clinical symptoms of border disease, have been evaluated by the palindromic nucleotide substitution (PNS) method at the three variable loci (V1, V2 and V3) in the 5′-untranslated region (UTR) of genomic RNA. The characteristic two base-pairings common to the BVDV-2 species, a C-G pairing which was common to the V1 locus, and a G-U pairing common to the V2 locus, were observed in all tested strains. Strains BD-78 and C413 were identified by a unique C-G pairing at position 4 from the bottom of the V2 stem region, which is characteristic to BVDV-2b. BVDV-2d characteristic U-A pairing at position 18 of the V1 stem region was observed in five strains, Lees, 167 237, 168 149, 173 157 and 175 375. No strains have been assigned to the genotypes BVDV-2a or BVDV-2c. Furthermore, the investigation at the level of the 5′-UTR excluded the application in sheep of the proposed BVDV-2 genetic virulence markers described in cattle. The two specific positions of uracil and cytosine nucleotides related to low or high virulence where indifferently present in the ovine BVDV-2 strains responsible of border disease.

KEY WORDS: BVDV-2, genotype, pestivirus.

Bovine viral diarrhea virus 2 (BVDV-2), represents an established species of the genus Pestivirus of the family Flaviviridae, with Flaviviridae

of the 5′-UTR can be divided into four domains, A-D, with domain D encompassing the two thirds in the 3′ region of the 5′-UTR predicted to fold into a complex palindromic stem-loop structure [5, 9], a critical region of the 5′-UTR, which is responsible for translational, transcriptional and replicational events in pestiviruses. Therefore, random mutations at the 5′-UTR have a high probability of incompatibility with viral survival. Thus stable nucleotide variations at this level assume high importance in terms of virus evolutionary history. Nucleotide sequences at the three variable loci, V1, V2 and V3, in the 5′-UTR of pestiviruses have been shown to be palindromic and capable of forming a stable stem-loop structure peculiar to each Pestivirus species [11]. Nucleotide substitutions in the stem regions always occur to maintain the palindromic sequence and thereby form a stable stem-loop structure. Thus, this type of mutation is referred to palindromic nucleotide substitutions (PNS). Based on the above mentioned considerations, the observation of nucleotide variations among virus strains at the level of the three specific palindromes in the 5′-UTR has been conceived as method for genotyping [11]. The results of the PNS method are essentially qualitative and provide the exact species classification of an isolate, although they offer no percentage values for homology among species and genotypes. According to the PNS method, the BVDV-1 species have been segregated into fourteen genotypes [10, 15, 16]. The taxonomic status of a giraffe strain was characterized, based on the 5′-UTR, as a new cluster among Pestivirus species [12]. The application of the PNS method to the BVDV-2 species showed four genotypes, BVDV-2a, BVDV-2b, BVDV-2c and BVDV-2d [14].

Among BDV strains previously classified depending on the host animal species, occurrence of other Pestivirus species has long been suspected [4, 6, 19]. Technical terms
BVDV-1 and BVDV-2 infections may occur with strains deposited in the nucleotide sequence databases have been found to be BVDV-1 or BVDV-2 by the PNS method [13]. BVDV-1 and BVDV-2 infections may occur with mild clinical symptoms or lead to subclinical courses in sheep. The similarity of clinical disease caused by BDV showed the utility of the PNS genetic characterization for laboratory diagnosis of the border disease in sheep. Since definition of an infectious disease is based primarily on a specific causative pathogen, clinical cases in sheep showing symptoms referring to border disease should be named according to the laboratory results. The number of ovine BVDV-2 isolates, reported until now, is limited. In addition, it is not excluded that the five ovine strains reported by Vilcek et al. [26] are contaminants from fetal calf serum during cultivation of cells [24]. Therefore, particular attention is required in performing isolation on cell cultures. RT-PCR assay for the rapid recognition of virus in blood sample or original tissue homogenates with no cultivation on cells [24] was apparent in other Pestivirus species. In a recent study, twenty-two (41.5%) out of 53 strains of ovine pestivirus isolates deposited in the nucleotide sequence databases have been found to be BVDV-1 or BVDV-2 by the PNS method [13]. BVDV-1 and BVDV-2 infections may occur with mild clinical symptoms or lead to subclinical courses in sheep. The similarity of clinical disease caused by BDV showed the utility of the PNS genetic characterization for laboratory diagnosis of the border disease in sheep. Since definition of an infectious disease is based primarily on a specific causative pathogen, clinical cases in sheep showing symptoms referring to border disease should be named according to the laboratory results. The number of ovine BVDV-2 isolates, reported until now, is limited. In addition, it is not excluded that the five ovine strains reported by Vilcek et al. [26] are contaminants from fetal calf serum during cultivation of cells [24]. Therefore, particular attention is required in performing isolation on cell cultures. RT-PCR assay for the rapid recognition of virus in blood sample or original tissue homogenates with no cultivation on cells for border disease clinical cases could be a useful approach.

In the present study, eight BVDV-2 strains isolated from sheep showing clinical symptoms of border disease and one adventitious strain from ovine cell line were evaluated by the PNS analysis in the 5'-UTR in order to determine the correct appurtenance among genotypes in the species and verify the presence of genetic virulence markers. The nucleotide sequences in the 5'-UTR of most ovine pestiviruses were obtained from the DNA databases. These strains, as compared with reference strains of BVDV-1 and BVDV-2, BDV and CSFV, were allocated to BVDV-2 species [14]. Strains of the tested nucleotide sequences are as follows (accession number is given in parenthesis); strain C413 (AF002227) deposited by Chen and Berry (unpublished), strain BD-78 (U18330) [20], strains Lees (U65051), 167 237 (U65055), 168 149 (U65056), 173 157 (U65058), and 175 375 (U65059) deposited by Vilcek et al. [26]. These ovine strains were all isolated from outbreaks of border disease (Table 1). The secondary structures were predicted according to the algorithm of Zuker and Stiegler [27]. The minimum free energy was calculated by the method of Freier et al. [8]. Three variable regions, V1, V2 and V3, at the 5'-UTR were used for genotyping based on the PNS [11].

All the tested strains shared the characteristic two base pairings common to BVDV-2 species, a C-G pairing which was common to the V1 locus, and a G*U pairing common to the V2 locus. The isolates have been classified within BVDV-2 by the PNS method into two genotypes, BVDV-2b, and BVDV-2d. No strains have been assigned to the genotypes BVDV-2a and BVDV-2c. In Fig. 1 are shown the V1, V2 and V3 palindromic loci in the 5'-UTR of the BVDV-2 species. Out of seven strains tested, two strains, BD-78 and C413, belonged to the BVDV-2b and five strains, Lees, 167 237, 168 149, 173 157 and 175 375, belonged to the BVDV-1d. Strains BD-78 and C413 were identified by a unique C-G pairing at position 4 from the bottom of the V2 stem region, characteristic to BVDV-2b. BVDV-2d characteristic U-A pairing at position 18 of the V1 stem region was observed in five strains, Lees, 167 237, 168 149, 173 157 and 175 375. The BVDV-2a specific combination of three base pairings (A-U in position 1 and Y:G in position 18 in V1 and U:R in position 4 in V2) and the BVDV-2c specific base pairing G*U at the bottom of the V1 stem were not apparent in any of the evaluated ovine strains.

In the present study, the genotyping procedure according to PNS in the 5'-UTR of the genomic RNA sequence of ovine BVDV-2 isolates completed their characterization, identifying two clusters among the four described genotypes in the BVDV-2 species. The BVDV-2 strains could be genetically differentiated, in terms of specific nucleotide substitutions, from the BVDV species. BVDV-2 shared specific base pairing common to the V1 and V2 loci. Four genotypes, BVDV-2a, BVDV-2b, BVDV-2c and BVDV-2d, were classified within BVDV-2 by the PNS method (Fig. 1). BVDV-2a genotype was identified by a specific combination of three base pairings. BVDV-2b was identified by a unique base pairing in V2 stem region. BVDV-2c and BVDV-2d were recognized by a pairing in the V1 stem.

A further application of genetic investigation in the 5'-

Table 1. Genotypes of Bovine viral diarrhea virus 2 strains isolated from sheep

<table>
<thead>
<tr>
<th>Strain</th>
<th>Virulence degree</th>
<th>Accession</th>
<th>Genotype</th>
<th>N1*</th>
<th>N2</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD-78</td>
<td>Border disease</td>
<td>U18330</td>
<td>BVDV-2b</td>
<td>U</td>
<td>C</td>
</tr>
<tr>
<td>C413</td>
<td>Border disease</td>
<td>AF002227</td>
<td>BVDV-2b</td>
<td>U</td>
<td>C</td>
</tr>
<tr>
<td>Lees</td>
<td>Border disease</td>
<td>U65051</td>
<td>BVDV-2d</td>
<td>C</td>
<td>U</td>
</tr>
<tr>
<td>167 237</td>
<td>Border disease</td>
<td>U65055</td>
<td>BVDV-2d</td>
<td>C</td>
<td>U</td>
</tr>
<tr>
<td>168 149</td>
<td>Border disease</td>
<td>U65056</td>
<td>BVDV-2d</td>
<td>C</td>
<td>U</td>
</tr>
<tr>
<td>173 157</td>
<td>Border disease</td>
<td>U65058</td>
<td>BVDV-2d</td>
<td>C</td>
<td>U</td>
</tr>
<tr>
<td>175 375</td>
<td>Border disease</td>
<td>U65059</td>
<td>BVDV-2d</td>
<td>C</td>
<td>U</td>
</tr>
</tbody>
</table>

N1 and N2 represent nucleotides, U (uracil) or C (cytosine), responsible for virulence markers proposed by Topliff and Kelling [23].
Fig. 1. Three palindromic loci V1, V2 and V3 at the 5'-UTR of the BVDV-2 species. Base-pairings characteristic to the genus (genus specific PNS) are shown in brown. The characteristic base-pairings of the BVDV-2 species (species specific PNS) are represented in blue, and genotype-specific PNS are shown in red. Watson-Crick base-pairings are indicated by a dash (–); tolerated pairings in secondary structure are indicated by an asterisk (*); interchangeable base pairings are indicated by a column (:). R = A or G; W = A or U; Y = C or U; B = C or G or U. Nucleotide U conserved in the loop region of genotypes BVDV-2b and BVDV-2d are shown in green. Nucleotides related to virulence degree in BVDV-2 strains are indicated as N1 and N2, equivalent to nucleotide 219 and 278, respectively, according to the Osloss strain.
UTR was based on the similarity of the secondary structure with the IRES of Poliovirus [5] in which nucleotide substitutions were supposed to be responsible for the virulence. Mutations or nucleotide substitutions at this region will affect efficiency of translation. Thus low translation will reduce virulence, and high translation increase virulence. Due to genetic and structural similarities with pestiviruses, this concept was proposed on BVDV-2 by Topliff and Kelling [22]. These authors reported a relatedness between high virulence and two specific nucleotides, uracil and cytosine located at position 219 and 278 of the genomic sequence (based on strain Osloss) and low virulence with cytosine and uracil at position 219 and 278, respectively. The virulence marker nucleotides were located at the V1 loop (for nucleotide 219) and V2 stem (for nucleotide 278) regions in IRES. The N1 nucleotide corresponded to position 8 in the V1 loop after the cytosine of the first C-G base-pair, characteristic C_C bulge. The N2 nucleotide corresponded to position 1 after the cytosine of the first C-G base-pair, characteristic to the genus Pestivirus, in the V2 stem and position 5 before the first G at the V2 loop sequence, GGGGU. The two specific uracil and cytosine nucleotides where indifferently present in the ovine BVDV-2 strains responsible of border disease. In the strains isolated from sheep with symptoms of border disease, the presence of cytosine in position 219 and uracil in position 278 was the most frequent in strains Lees, 167 237, 168 149, 173 157 and 175 375 out of seven strains examined. The presence of uracil in position 219 and cytosine in position 278, which corresponded to the supposed virulence markers, was only observed in the two strains BD-78 and C413 of BVDV-2b. However, also in bovine viruses, nucleotide changes do not always seem determinative for virulence markers. The hyper-virulent strains BVDV-2c AZ Spl [18] and BVDV-2a CD87 [17] showed cytosine and uracil in positions corresponding to low virulence [13]. Thus these two nucleotides, N1 and N2, seem to be rather meaningless in virulence.

The PNS analysis in the 5'-UTR demonstrated a rationale and simple approach for viral investigations. Secondary structures predicted at the three variable regions in the 5'-UTR showed typical PNS which were useful for classification or genotyping of ovine Pestivirus strains. Monitoring of BVDV-2 infection in sheep with genetic characterization of viral types or subtypes based on genetic changes could provide useful information and improve our understanding of virus epizootiology and evolutionary history in small ruminants and the relationship with cattle and other susceptible animals and might provide markers for biological differences, such as in virulence. Nevertheless, the control of the BVDV-2 infection in sheep might be an indirect preventive measure against the potential for severe syndromes characterized by thrombocytopenia and high mortality in cattle. The risk of iatrogenic infection and the occurrence of immune-tolerant, carrier animals, should also be considered.

REFERENCES