Variability of E2 protein-coding sequences of bovine viral diarrhea virus in Polish cattle

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Abstract

Bovine viral diarrhea virus (BVDV) belongs to the Pestivirus genus of the Flaviviridae family and has worldwide distribution, being one of the main causes of economic losses in cattle raising. The genome of pestiviruses is a single strand of positive-sense RNA with a length of 12.3 kb, which encodes one open reading frame flanked by untranslated regions. E2 glycoprotein is required for binding to cell-surface receptors and it also contains major antigenic determinants. The nucleotide sequence coding E2 is the most variable part of the viral genome. The heterogeneity that exists among circulating strains causes problems in the development of effective vaccines and reliable diagnostics. In this study, and for the first time analysis was made of the E2 glycoprotein coding sequences of 14 Polish BVDV-1 strains which belong to four subtypes: 1b (n = 7), 1f (n = 3), 1s (n = 3), and 1r (n = 1). These sequences showed evidence of strong purifying (negative) selection. However, we also identified positively selected sites. The availability of E2 sequences of Polish BVDV strains for reference, knowledge gained through epitope prediction attempts, and information on protein glycosylation sites can afford a better understanding of host–pathogen interactions.

Keywords  Bovine viral diarrhea virus · E2 glycoprotein · Pestivirus · Polymorphism · Sequencing

Bovine viral diarrhea virus (BVDV) is one of the most widespread viruses in cattle breeding and causes large economic losses [1]. In Europe, the prevalence of antibody-positivity in animals in countries without systemic BVD control is between 60 and 85% [2]. BVDV can induce many different clinical symptoms in infected animals. Usually, these symptoms are mild, although hemorrhagic syndrome and mucosal disease can manifest in the rare severe cases [3]. BVDV species 1 (BVDV-1, Pestivirus A) and 2 (BVDV-2, Pestivirus B) belong to the Pestivirus genus of the Flaviviridae family together with classical swine fever virus (CSFV, Pestivirus C) and border disease virus (BDV, Pestivirus D) [4]. The genetic material of pestiviruses is a single-stranded positive-sense RNA with a length of 12.3 kb, which encodes one open reading frame flanked by untranslated regions from both ends.

A virion has a diameter of 40–60 nm and it is surrounded by a lipid membrane. The lipid membrane contains three glycosylated envelope proteins: E1, E1, and E2 [5]. The two envelope glycoproteins E1 and E2 recognize host cells by binding to cell-surface receptors CD46 and LDL-R [6] and are required for membrane fusion and cell entry [7]. E1 is assumed to function as a membrane anchor for E2 [8], which also contains major antigenic determinants [9, 10]. The BVDV genome has a high mutation rate and the E2 glycoprotein coding fragment is the most variable part of its genome. The heterogeneity that exists among circulating strains causes problems in the development of effective vaccines and sensitive diagnostics.

The present study is the first to provide sequence information for the E2 glycoprotein of Polish BVDV strains belonging to the most often isolated subtypes in Poland. We focused on determining which E2 glycoprotein regions are subject to positive selection and the detection of protein glycosylation sites. This data may be a key indicator of the nature of the host–virus interaction. In this study, we used BVDV-positive serum samples from previous detection and genotyping studies of clinical suspects, herds with
virus eradication underway and herds vaccinated with killed BVDV-1a vaccine [11].

Total RNA was extracted using TRI Reagent (Sigma-Aldrich, USA) from 500 µL of serum following the manufacturer’s instructions. Reaction mixes for standard RT-PCR were prepared as described previously [11]. A mix of four primer pairs specific to the E2-encoding fragment [12] and specific to regions flanking the E2 encoding sequence [13–15] was used. A list of primer sequences is presented in Table 1. We obtained positive RT-PCR results for 16 out of 30 samples in the form of a band on agarose gel with a size of about 1019–1200 nucleotides. However, for further studies, it was only possible to use 14 viral sequences, as only this many were of good quality, and these were submitted to GenBank with the accession numbers MK675059–MK675072. The list of strains for which sequences were obtained can be found in Table 2, where the geographical origin of the samples is given. The analyzed sequences were assigned to four groups on the phylogenetic tree (Fig. 1), to the same subtypes as in the previous study [11]. Subtype 1b is currently the most often isolated subtype of BVDV in Poland. Almost a quarter of all isolated viruses belonged to the 1f subtype. The remaining two subtypes, 1r and 1s, were identified recently and are rare.

We were able to amplify the E2 region from a much smaller number of strains than was possible for the 5′ untranslated region in a previous study [11]. Due to the high variability, the E2 region is not suitable as a target for diagnostic purposes, but it can provide useful information when results from the 5′UTR are unclear. The analyzed fragment of the E2 region is longer than 5′UTR, it is less conserved, and the constructed phylogenetic tree within this region has higher bootstrap values.

The percentage of sequence identity of our strains was calculated based on a 892-nucleotide fragment (nucleotide positions 55–946 and amino acid positions 19–315 of the C86 vaccine strain in the E2 gene). The fragment analyzed is shorter than the full-length E2 sequence due to unsatisfactory sequence quality at its ends. The identity between various strains ranged from 70.4 to 98.5%, with the smallest differences occurring between subtypes 1f and 1s (79.5–81.75% identity), and the largest ones between 1b and 1f (70.4–73% identity). Differences among strains within subtypes 1b, 1s, and 1f were 84.7–96%, 94.2–98.5%, and 83.9–98.5%, respectively. The analyzed region of E2 displayed evolutionary distances of 1.5–16.1% at the subtype level and 18.3–29.6% among subtypes. Similar evolutionary distances to the Polish strains were also found among Chinese strains [16]. The E2 sequences from this study displayed 437/892 (48.9%) variable sites at the nucleic acid level: 83 singleton variable sites and 354 parsimony informative sites. The total number of mutations was 597. We identified 43.77% variable sites at amino acid level, which presented up to six amino acid variants, and these six variants occurred in positions 57, 159, and 198. Five variants appeared in 13 different positions.

We investigated the nature of selection pressure acting on the envelope glycoprotein E2 gene by the estimation of synonymous/nonsynonymous (dS/dN) mutation rates (ω) using DNASP6.11 [17]. The E2 glycoprotein coding region showed evidence of strong purifying selection throughout the sequence (ω = 0.143), i.e., stabilizing it through the removal of deleterious genetic polymorphisms that arise through random mutations. Positive selection is a type of natural selection in which a specific phenotype is preferred to other phenotypes. We have identified several locations in the genome that are characterized by positive selection (Fig. 1): 68–70 (dN/dS ratio 1.893), 91–96 (1.087), 163–165 (2.212), 170–173 (1.767), 187–189 (1.232), 286–288 (4.818), 415–417 (1.552), 454–456 (3.339), 475–477 (2.972), and 754–756 (3.301).

E2 glycoprotein, which is the most immunodominant viral protein of pestiviruses, contains major type-specific epitopes recognized by specific antibodies. This protein is presented by antigen-presenting cells and was identified as a target for cytotoxic T-cells [18]. Positive selection might contribute to the avoidance of T-cell recognition of the E2 glycoprotein. For BVDV-1, essential amino acid positions for neutralization by monoclonal antibodies (mAbs) have been mapped in the N-terminal part of E2 [19]. Two antigenic regions of the E2 glycoprotein were mapped between

| Primer | Sequence 5′–3′ | Reference |
|--------|----------------|-----------|
| F1     | AGCAGCTGAGGGGCAACTAAT | Pecora et al. [15] |
| R1     | GCTGACATGACTAATCTCTCAGT |  |
| R2     | TTCATTTCACCTGCAGACCC |  |
| 738F   | TRGGCTGCTACTAGTACNCGGCCACAAGG | van Rijn et al. [13] |
| 810F   | TGCTACTACTAGTACNCGGTTCAAGG |  |
| BVIIIR | GTRGCAATTGTCGGCCTCATYAC | Tijssen et al. [14] |
| 2274F  | TGTTGCCCTATGAGAC | Nagai et al. [12] |
| 3434R  | AGGTCAAACCAARTTGG |  |
### Field strains for which the sequences of the E2 region were obtained and reference strains retrieved from GenBank for comparative analysis

| Species | Herd | Strain     | Subtype | Region                        | Vaccination | Accession number |
|---------|------|------------|---------|-------------------------------|-------------|------------------|
| BVDV-1  | 1    | 164-DM/15  | If      | Lublin Voivodeship            | No          | MK675059         |
| BVDV-1  | 1    | 165-DM/15  | If      |                               | No          | MK675060         |
| BVDV-1  | 2    | 166-KY/15  | Is      | Kuyavian-Pomeranian Voivodeship| No          | MK675061         |
| BVDV-1  | 2    | 167-KY/15  | Is      |                               | No          | MK675062         |
| BVDV-1  | 3    | 176-KR/15  | Is      |                               | No          | MK675063         |
| BVDV-1  | 4    | 177-EP/16  | Ib      | Lublin Voivodeship            | No          | MK675064         |
| BVDV-1  | 5    | 179-WD/17  | If      | Lublin Voivodeship            | No          | MK675065         |
| BVDV-1  | 6    | 183-SY/17  | Ir      | Świętokrzyskie Voivodeship     | No          | MK675066         |
| BVDV-1  | 7    | 186-km/17  | Ib      | Wielkopolska Voivodeship      | No          | MK675067         |
| BVDV-1  | 8    | 187-AN/17  | Ib      | Wielkopolska Voivodeship      | Yes         | MK675068         |
| BVDV-1  | 9    | 194-TC/17  | Ib      | Wielkopolska Voivodeship      | Yes         | MK675069         |
| BVDV-1  | 10   | 200-BA/17  | Ib      | Wielkopolska Voivodeship      | No          | MK675070         |
| BVDV-1  | 11   | 207-LK/18  | Ib      | Wielkopolska Voivodeship      | No          | MK675071         |

| Species       | Strain | Subtype | Accession number |
|---------------|--------|---------|------------------|
| BVDV-1 NADL   |        | 1a      | M31182.1          |
| BVDV-1 Singer |        | 1a      | DQ088995.2        |
| BVDV-1 C86    |        | 1a      | Y19123.1          |
| BVDV-1 Oregon C24V |   | 1a      | AF091605.1        |
| BVDV-1 VEDEVAC |       | 1b      | AJ585412.1        |
| BVDV-1 Osloss |        | 1b      | M96687.1          |
| BVDV-1 KE9    |        | 1b      | EF101530.1        |
| BVDV-1 New York-1 (NY-1) | | 1b | AY027671.1 |
| BVDV-1 XZ02   |        | 1b      | MF278652.1        |
| BVDV-1 Braidwood |      | 1c      | AF255049.1        |
| BVDV-1 Bega   |        | 1c      | AF049221.2        |
| BVDV-1 519    |        | 1c      | AF144610.1        |
| BVDV-1 10JJ-SKR |      | 1d      | KC757383.1        |
| BVDV-1 BJ1308 |        | 1d      | KT951841.1        |
| BVDV-1 SLO/2407/2006 | | 1e | KX577637.1 |
| BVDV-1 Carlito |       | 1e      | KP313732.1        |
| BVDV-1 SLO/1170/2000 |  | 1f      | KX987157.1        |
| BVDV-1 UM/103/04 |    | 1g      | LT797813.1        |
| BVDV-1 UM/12607 |       | 1h      | LT631725.1        |
| BVDV-1 ACM/BR/2016 |   | 1i      | KX857724.1        |
Table 2 (continued)

Reference strains

| Species | Strain     | Subtype | Accession number |
|---------|------------|---------|------------------|
| BVDV-1  | KS86-1ncp  | lj      | AB078950.1       |
| BVDV-1  | KS86-1cp   | lj      | AB078952.1       |
| BVDV-1  | SuwaCP     | 1k      | KC853441.1       |
| BVDV-1  | SuwaNeP    | 1k      | KC853440.1       |
| BVDV-1  | ZM-95      | 1m      | AF526381.3       |
| BVDV-1  | SD-15      | 1m      | KR866116.1       |
| BVDV-1  | Shiara/02/06 | 1n   | LC089876.1       |
| BVDV-1  | IS26/01ncp | 1o      | LC089875.1       |
| BVDV-1  | TJ41       | 1o      | KF048848.1       |
| BVDV-1  | LE101      | 1p      | KF048849.1       |
| BVDV-1  | TJ142      | 1p      | KF048850.1       |
| BVDV-1  | camel-6    | 1q      | KC695810.1       |
| BVDV-1  | GS-3       | 1q      | KC695811.1       |
| BVDV-1  | VE245/12   | 1r      | LT837585.1       |
| BVDV-1  | Mousedeer  | 1s      | AY162456.1       |
| BVDV-2  | New York-93 (NY-93) | 2a | KR093034.1       |
| BVDV-2  | 793        | 2a      | HQ174302.2       |
amino acid positions 1–70 and 70–77 [20]. In this study, we identified five sites showing positive selection in the 1–70 fragment. Other studies showed that single nucleotide mutation causing the change of one of the four amino acids in the 71–74 region can defeat neutralization by a single mAb [20]. Changes at position 72 observed in Singer BVDV-1a strain mutants affected the binding and neutralization of mAbs 157 and 348 [20]. Leucine at position 74 was also important for the binding of the WB166 antibody [21]. The Singer strain showing a different change, at position 32, managed to evade the antibody 157. Similarly, the Hastings strain of BVDV-1 also managed to escape this antibody through a change in the same position [20]. We identified mild positive selection for this position in Polish strains of BVDV-1.

An immunodominant region at amino acid position 121–150, corresponding to the linear neutralizing epitope of the E2 protein of CSFV, was recognized by polyclonal antibodies [22]. There were differences in nine positions in our strains, particularly in the C-terminal part. We also observed a positive selection in this region, specifically for position 139.

Amino acids 96 and 152 showed the strongest positive selection of the strains from this study. However, identifying the importance of this selection requires further research.

The sequence coding for the receptor binding domain, which usually mediates binding of the virion to the host cell surface, is located at amino acid position 141–170 of E2 in CSFV, indicating that binding of the receptor CD46 might occur through this region [23]. It corresponds to the polymorphic 142–172 segment in the BVDV genome. We did not detect positive selection in the area of the receptor binding site, but high variability within a protein having a cell receptor affinity may contribute to a change in tropism.

![Phylogenetic tree](image)  
**Fig. 1 a** Phylogenetic tree based on a 636 nt fragment of the E2 gene. Black dots indicate analyzed strains. **b** Consensus sequence of tested strains with positive selection sites and glycosylation sites.
This would be caused by random mutations enabling the crossing of species barriers and possible infection of a wider range of hosts within even-toed ungulates (Artiodactyla).

Four glycosylation sites in the E2 glycoprotein predicted by NetNGlyc 1.0 have been identified in positions N117, N186, N230, and N298. One extra glycosylation site in position N25 (Fig. 1) was found in the 164-DM/15 and 165-DM/15 strains (subtypes 1f) detected in samples from the same herd (Table 2). N-linked glycosylation is crucial in protein functions, such as entry into host cells, protein antigenic properties, proteolytic processing, and protein trafficking [24].

Strains 187-AN/17 and 194-TC/17 were isolated from vaccinated animals (Table 2). The vaccine was based on the C86 strain (GenBank accession number Y19123) belonging to subtype 1a. The percentage of nucleotide sequence identity of the tested field strains and the C86 strain sequence taken from the GenBank database was 72.6–73.2% and at the protein level it was 75.4–75.7%.

BVDV vaccines are mostly based on single subtypes including BVDV-1a, BVDV-1b, or BVDV-2a. Our and other investigators’ results indicate that current vaccines based on a limited number of subtypes do not provide effective protection against other subtypes of BVD virus [25]. Humoral immune response directed against E2 is considered the main line of defense against the BVD virus circulating in the field [19]. Sequence identity between our strains and the vaccine strain C86 in the 19–90 amino acid position where many antibody-recognizable epitopes was only 55.5–63.8%. In this fragment sequence similarity percentage between our strains and the C86 vaccine strain is even lower than in the entire E2 sequence studied by us.

In summary, the present study provides information for the first time of sequences of the E2 glycoprotein of Polish BVDV strains belonging to the most often isolated subtypes in Poland. We showed that E2 glycoprotein with its high genetic variability contains fragments that are positively selected. Some of these fragments may be epitopes. We believe that the strain used in vaccine production should show greater similarity to strains circulating in a given region, and that particular emphasis should fall on similarity at sites encoding immunogenic epitopes present mainly in the E2 glycoprotein. In contrast to the most frequently studied 5′UTR and Npro region, the positively selected BVDV sites require further analysis to establish if amino acid substitutions can lead to changes of host tropism, evasion of epitope-specific CD8 T-cell response, or avoidance of antibody recognition. Understanding the importance of amino acid changes at sites of positive selection can be helpful in studying the virulence of strains and predicting future responses to vaccine strains of BVDV.

Author contributions PM conducted and coordinated the study including laboratory and computer analysis and drafted the manuscript. MP drafted and revised the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest We declare that all authors have no conflict of interests.

Ethical approval This article does not contain any studies on animals performed by any of the authors.

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References

1. Houe H (2003) Economic impact of BVDV infection in dairies. Biologicals 31:137–143
2. Houe H (1999) Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. Vet Microbiol 64:89–107
3. Bachofen C, Braun U, Hilbe M, Ehresperger F, Stalder H, Peterhans E (2010) Clinical appearance and pathology of cattle persistently infected with bovine viral diarrhoea virus of different genetic subgroups. Vet Microbiol 141:258–267
4. Simmonds P, Becher B, Bukh J, Gould EA, Meyers G, Monath T, Muerhoff S, Pletnev A, Rico-Hesse R, Smith DB, Stapleton JT (2017) ICTV report consortium, ICTV virus taxonomy profile: Flaviviridae. J Gen Virol 98:2–3
5. Wegelt A, Reimann I, Granzow H, Beer M (2011) Characterization and purification of recombinant bovine viral diarrhoea virus particles with epitope-tagged envelope proteins. J Gen Virol 92:1352–1357
6. Krey T, Himmelreich A, Heimann M, Menge C, Thiel HJ, Maurer K, Rümenapf T (2006) Function of bovine viral diarhoea virus is determined by complement control protein 1. J Virol 80(8):3912–3922
7. Ronecker S, Zimmer G, Herrler G, Greiser-Wilke I, Grummer B (2008) Formation of bovine viral diarrhoea virus E1–E2 heterodimers is essential for virus entry and depends on charged residues in the transmembrane domains. J Gen Virol 89:2114–2121
8. Rümenapf T, Unger G, Strauss JH, Thiel HJ (1993) Processing of the envelope glycoproteins of pestiviruses. J Virol 67:3288–3294
9. Deregt D, van Rijn PA, Wiens TY, van den Hurk J (1998) Monoclonal antibodies to the E2 protein of a new genotype (type 2) of bovine viral diarrhea virus define three antigenic domains involved in neutralization. Virus Res 57:171–181
10. Ridpath JE, Neill JD, Endsley J, Roth JA (2003) Effect of passive immunity on the development of a protective immune response against bovine viral diarrhea virus in calves. Am J Vet Res 64:65–69
11. Mirosław P, Polak M (2019) Increased genetic variation of bovine viral diarrhea virus in dairy cattle in Poland. BMC Vet Res 15(1):278
12. Nagai M, Hayashi M, Sugita S, Sakoda Y, Mori M, Murakami T, Ozawa T, Yamada N, Akashi H (2004) Phylogenetic analysis of bovine viral diarrhea viruses using five different genetic regions. Virus Res 99:103–113
13. van Rijn PA, van Gennip HG, Leendertse CH, Bruschke CJ, Paton DJ, Moormann RJ, van Oirschot JT (1997) Subdivision of the Pestivirus genus based on envelope glycoprotein E2. Virology 237:337–348
14. Tijssen P, Pellerin C, Lecomte J, van den Hurk J (1996) Immunodominant E2 (gp53) sequences of highly virulent bovine viral diarrhea group II viruses indicate a close resemblance to a subgroup of border disease viruses. Virology 217:356–361
15. Pecora A, Malacari DA, Ridpath JF, Aguirreburualde Perez MS, Combessies G, Odeón AC, Romera SA, Golemba MD, Widgorovitz A (2013) First finding of genetic and antigenic diversity in 1b-BVDV strains from Argentina. Res Vet Sci 96:204–212
16. Lang YF, Gao SD, Du JZ, Shao JJ, Cong GZ, Lin T, Zhao FR, Liu LH, Chang HY (2014) Polymorphic genetic characterization of E2 gene of bovine viral diarrhea virus in China. Vet Microbiol 174:554–559
17. Rozas J, Ferrer-Mata A, Sánchez-DelBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE, Sánchez-Gracia A (2017) DnaSP 6: DNA sequence polymorphism analysis of large datasets. Mol Biol Evol 34:3299–3302
18. Ceppi M, de Bruin MG, Seuberlich T, Balmelli C, Pascolo S, Ruggli N, Wienholt D, Tratschin JD, McCullough KC, Summerfield A (2005) Identification of classical swine fever virus protein E2 as a target for cytotoxic T cells by using mRNA-transfected antigen-presenting cells. J Gen Virol 86:2525–2534
19. Paton DJ, Lowings JP, Barrett AD (1992) Epitope mapping of the gp53 envelope protein of bovine viral diarrhea virus. Virology 190:763–772
20. Deregt D, Bolin SR, van den Hurk J, Ridpath JF, Gilbert SA (1998) Mapping of a type 1-specific and a type-common epitope on the E2 (gp53) protein of bovine viral diarrhea virus with neutralization escape mutants. Virus Res 53:81–90
21. Jelsma H, Loeffen WL, van Beuningen A, van Rijn PA (2013) Preliminary mapping of non-conserved epitopes on envelope glycoprotein E2 of bovine viral diarrhea virus type 1 and 2. Vet Microbiol 166:195–199
22. Lin M, Lin F, Mallory M, Clavijo A (2000) Deletions of structural glycoprotein E2 of classical swine fever virus strain Alfort/187 resolve a linear epitope of monoclonal antibody WH303 and the minimal N-terminal domain essential for binding immunoglobulin G antibodies of a pig hyperimmune serum. J Virol 74:11619–11625
23. Li X, Wang L, Zhao D, Zhang G, Luo J, Deng R, Yang Y (2011) Identification of host cell binding peptide from an overlapping peptide library for inhibition of classical swine fever virus infection. Virus Genes 43(1):33–40
24. Vigerust DJ, Shepherd VL (2007) Virus glycosylation: role in virulence and immune interactions. Trends Microbiol 15(5):211–218
25. Newcomer BW, Chamorro MF, Walz PH (2017) Vaccination of cattle against bovine viral diarrhea virus. Vet Microbiol 206:78–83

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