Increased genetic variation of bovine viral diarrhea virus in dairy cattle in Poland

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Abstract

Background: Bovine viral diarrhea virus (BVDV) causes severe economic losses and is one of the most important viral pathogens of ruminants worldwide. The infection manifests itself in a variety of clinical symptoms. Phylogenetic studies based mainly on 5'UTR of its genome, identified many different subtypes of BVDV. Previous study indicated the predominance of BVDV-1b and BVDV-1d in Poland. The aim of this study was to genotype BVDV isolates currently circulating in Polish dairy herds.

Results: BVDV was detected in 30 herds. Viral subtypes were identified using sequences of the 5'UTR fragment and they were confirmed within a fragment of the Npro region. Seven subtypes of BVDV-1 species have been identified: 1b, 1g, 1f, 1d, 1r, 1s and 1e.

Conclusion: The number of subtypes of BVDV in Poland evolves and 2 new subtypes have been identified for the first time. Such studies may have a positive impact on successful eradication of the virus using effective vaccines and diagnostic tests.

Keywords: Bovine viral diarrhea virus, Genetic diversity, Subtypes, Pestivirus, Cattle

Background

Bovine viral diarrhea virus (BVDV) belongs to Pestivirus genus in the Flaviviridae family [1]. It consists of four recognized species: bovine viral diarrhea virus type 1 (BVDV-1, Pestivirus A), type 2 (BVDV-2, Pestivirus B), classical swine fever virus (CSFV, Pestivirus C) and border disease virus (BDV, Pestivirus D). A few putative species have been discovered recently which may be classified as members of the Pestivirus genus but they have not been approved as species yet. Among them are: HoBi-like pestiviruses (also called BVDV-3) identified first in batches of contaminated foetal calf serum [2] and then in calves and aborted fetuses [3, 4], giraffe pestivirus associated with the outbreak of mucosal-like disease in Kenyan giraffes [5], Bungowannah virus detected in pig herds in Australia where stillbirth foetuses and neonatal deaths were observed [6] and Pronghorn virus, isolated from a pronghorn antelope in the United States [7]. There are also reports of novel pestiviruses in other animal species like rats and bats [8, 9]. This wide range of pestiviruses infecting different animal species is the proof of genetic plasticity of their genomes, adapting to different hosts.

BVDV is an important pathogen of cattle worldwide with significant economic impact [10]. Infection may lead to a wide array of clinical signs from subclinical to severe acute hemorrhagic syndrome and fatal mucosal disease [11]. BVDV also causes immunosuppression, which increases the severity of clinical picture when other pathogens are involved. BVDV infection of seronegative and pregnant females during the first 40–120 days of pregnancy may lead to the birth of persistently infected (PI) calves. They remain infected for life and shed the virus in high titre, ensuring the persistence of BVDV in the herd if they are not removed immediately after identification.

Viral genome is comprised of a single-stranded positive sense RNA about 12.3 kb in size with one large open reading frame flanked by 5' and 3' untranslated regions (5'UTR and 3'UTR respectively) [1]. Pestiviral genome encodes a single polyprotein that is processed into either 11 or 12 proteins: Npro, C, Ems, E1, E2, p7, NS2–3 (NS2, NS3), NS4A, NS4B, NS5A, NS5B. Several regions of BVDV genome have been used to study its genetic diversity [12, 13]. Phylogenetic analysis is mostly based on the...
comparison of nucleotide sequences from the 5'UTR, N\textsuperscript{pro} or E2 regions of viral genome. Based on genetic studies, 21 subtypes of BVDV-1 (1a - 1u) and 4 subtypes of BVDV-2 (2a – 2d) were identified so far [14, 15]. BVDV-1 is the predominant pestivirus circulating in cattle population in Europe [16]. Similar situation was observed in Poland, where studies encompassing years 2004–2014 revealed the presence of five subtypes of BVDV-1: 1b, 1d, 1f, 1g [17] and 1e [18] in decreasing frequency. Later, BVDV-2a has been identified but only on one farm [19]. The aim of this study was to genotype BVDV isolates currently circulating in Poland. Such studies are important to understand epidemiology of the virus and they may support the development of successful control and eradication programs, where effective vaccines and reliable diagnostic tests are essential.

**Results**

Positive results in RT-PCR test for BVDV were obtained for 63 samples from 30 farms in all 8 provinces tested (overall prevalence of 0.7%). Nucleotide alignment with the reference strains from GenBank using BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that all detected strains were characterized as BVDV-1. For phylogenetic tree construction, a 208 nucleotide fragment of the 5’UTR was analyzed and final result with the genetic relatedness of field and reference strains is shown in Fig. 1. One isolate (213-GK/18) was sequenced only in the N\textsuperscript{pro} region (subtype 1f) therefore, sequence analysis in the 5’ untranslated region was based on 62 sequences. Field isolates were separated into seven groups representing seven separate subtypes. Twenty nine isolates were also genotyped within N\textsuperscript{pro} region. The phylogenetic tree of the N\textsuperscript{pro} was constructed based on a 281 nucleotide fragment (Fig. 2) fully confirming classification from 5’UTR even with higher bootstrap values. Analysis revealed that BVDV-1 strains belonged to subtypes 1b detected in 8 herds (n = 17), 1g in 8 herds (n = 17), 1f in 7 herds (n = 15), 1d in 3 herds (n = 6), 1r in 3 herds (n = 4), 1s in 2 herds (n = 3) and 1e detected in one herd (n = 1). In order to confirm the allocation of isolates to particular subtypes another tree was constructed using the Bayesian method (Additional file 1 and Additional file 2). Field strains have been assigned to the same subtypes. The list of analyzed isolates is given in Table 1. Animals from the same herd were infected with one subtype only and sequence homology between viral isolates at herd level was very high. The only exception were two farms: one in Wielkopolskie (Farm 10) and another one in Opolskie (Farm 29) province. After initial identification of BVDV-1d (184-KN/17, 185-KN/17, 196-KN/17) in Wielkopolskie farm, another subtype, namely BVDV-1g (206-KN/17) was identified in the same year. One year later in Opolskie province BVDV-1f was
Fig. 2 Phylogenetic tree based on NPRO fragment of 29 field isolates of BVDV. Black dots indicate field strains.
| Isolate      | Year of isolation | Farm | Sample | Region of isolation                  | Subtype | Accesion number |
|-------------|-------------------|------|--------|--------------------------------------|---------|----------------|
| 164-DM/15   | 2015              | 1    | Serum  | Lublin Voivodeship                   | 1f      | MK044822       |
| 165-DM/15   | 2015              | 1    | Serum  | Lublin Voivodeship                   | 1f      | MK044823       |
| 166-KY/15   | 2015              | 2    | Serum  | Kuyavian-Pomeranian Voivodeship      | 1s      | MK044824       |
| 167-KY/15   | 2015              | 2    | Serum  | Kuyavian-Pomeranian Voivodeship      | 1s      | MK044825       |
| 168-WS/15   | 2015              | 3    | Serum  | Wielkopolska Voivodeship             | 1g      | MK044826       |
| 169-WS/15   | 2015              | 3    | Serum  | Wielkopolska Voivodeship             | 1g      | MK044827       |
| 170-KR/16   | 2016              | 4    | Serum  | Wielkopolska Voivodeship             | 1g      | MK168328       |
| 171-SR/16   | 2016              | 4    | Serum  | Wielkopolska Voivodeship             | 1g      | MK168329       |
| 172-EP/16   | 2016              | 5    | Serum  | Lublin Voivodeship                   | 1b      | MK168330       |
| 173-EP/16   | 2016              | 5    | Serum  | Lublin Voivodeship                   | 1b      | MK168331       |
| 174-DS/15   | 2015              | 6    | Serum  | Wielkopolska Voivodeship             | 1d      | MK168332       |
| 175-DS/15   | 2015              | 6    | Serum  | Wielkopolska Voivodeship             | 1d      | MK168333       |
| 176-KR/15   | 2015              | 7    | Serum  | Kuyavian-Pomeranian Voivodeship      | 1s      | MK168334       |
| 177-EP/16   | 2016              | 5    | Serum  | Lublin Voivodeship                   | 1b      | MK168335       |
| 178-DM/15   | 2015              | 1    | Serum  | Lublin Voivodeship                   | 1f      | MK168336       |
| 179-WD/17   | 2017              | 8    | Serum  | Lublin Voivodeship                   | 1f      | MK381356       |
| 180-WD/17   | 2017              | 8    | Serum  | Lublin Voivodeship                   | 1f      | MK381357       |
| 181-WD/17   | 2017              | 8    | Serum  | Lublin Voivodeship                   | 1f      | MK381358       |
| 182-SY/17   | 2017              | 9    | Serum  | Świętokrzyskie Voivodeship           | 1r      | MK381359       |
| 184-KN/17   | 2017              | 10   | Serum  | Wielkopolska Voivodeship             | 1d      | MK381360       |
| 185-KN/17   | 2017              | 10   | Serum  | Wielkopolska Voivodeship             | 1d      | MK381361       |
| 186-KM/17   | 2017              | 11   | Serum  | Wielkopolska Voivodeship             | 1b      | MK381362       |
| 187-AN/17   | 2017              | 12   | Serum  | Wielkopolska Voivodeship             | 1b      | MK381363       |
| 188-LS/17   | 2017              | 13   | Serum  | Wielkopolska Voivodeship             | 1d      | MK381364       |
| 189-JA/17   | 2017              | 14   | Serum  | Wielkopolska Voivodeship             | 1d      | MK381365       |
| 190-JA/17   | 2017              | 14   | Serum  | Wielkopolska Voivodeship             | 1b      | MK381366       |
| 191-KW/17   | 2017              | 15   | Serum  | Łódź Voivodeship                     | 1f      | MK381367       |
| 192-KW/17   | 2017              | 15   | Serum  | Łódź Voivodeship                     | 1f      | MK381368       |
| 193-CZ/17   | 2017              | 16   | Serum  | Wielkopolska Voivodeship             | 1g      | MK381369       |
| 194-TC/17   | 2017              | 17   | Serum  | Wielkopolska Voivodeship             | 1b      | MK381370       |
| 195-RM/17   | 2017              | 18   | Serum  | Kuyavian-Pomeranian Voivodeship      | 1f      | MK381371       |
| 196-KN/17   | 2017              | 10   | Serum  | Wielkopolska Voivodeship             | 1d      | MK381372       |
| 197-BA/17   | 2017              | 19   | Serum  | Wielkopolska Voivodeship             | 1b      | MK381373       |
| 198-BA/17   | 2017              | 19   | Serum  | Wielkopolska Voivodeship             | 1b      | MK381374       |
| 199-BA/17   | 2017              | 19   | Serum  | Wielkopolska Voivodeship             | 1b      | MK381375       |
| 200-BA/17   | 2017              | 19   | Serum  | Wielkopolska Voivodeship             | 1b      | MK381376       |
| 201-BA/17   | 2017              | 19   | Serum  | Wielkopolska Voivodeship             | 1b      | MK381377       |
| 202-BA/17   | 2017              | 19   | Serum  | Wielkopolska Voivodeship             | 1b      | MK381378       |
| 203-TF/17   | 2017              | 20   | Serum  | Mazovian Voivodeship                 | 1g      | MK381379       |
| 204-TF/17   | 2017              | 20   | Serum  | Mazovian Voivodeship                 | 1g      | MK381380       |
| 205-TF/17   | 2017              | 20   | Serum  | Mazovian Voivodeship                 | 1g      | MK381381       |
| 206-KN/17   | 2017              | 10   | Serum  | Wielkopolska Voivodeship             | 1g      | MK381382       |
| 207-LK/18   | 2018              | 21   | Serum  | Wielkopolska Voivodeship             | 1b      | MK381383       |
identified (219-KH/18, 220-KH/18, 221-KH/18) followed by identification for the first time in Poland of BVDV-1r (218-KH/18, 222-KH/18) in the same farm. The number of isolates per farm was between 1 and 6, although at more than 80% of farms only 1 or 2 infected individuals were identified (Table 1). The number of subtypes identified annually was 4, 2, 5 and 5 in 2015, 2016, 2017 and 2018, respectively (Fig. 3). The most predominant subtypes of BVDV-1 per year were: 1f and 1 s (30% each) in 2015, 1b (60%) in 2016, 1b (41%) in 2017 and 1 g (38%) in 2018. The only subtype identified each year was BVDV-1 g while 1 s was identified only in 2015 (like 1e in 2018). Geographical clustering was observed for subtypes 1d, 1 s and 1e identified in different, single provinces. BVDV-1f was identified in 5 provinces, BVDV-1 g and BVDV-1r in 3 provinces, BVDV-1b in 2 provinces. The

| Isolate | Year of isolation | Farm | Sample | Region of isolation | Subtype | Accession number |
|---------|-------------------|------|--------|---------------------|---------|-----------------|
| 208-KT/18 | 2018 | 22 | Serum | Lublin Voivodeship | 1b | MK381384 MK381438 |
| 209-KT/18 | 2018 | 22 | Serum | Lublin Voivodeship | 1b | MK381385 MK381439 |
| 210-GK/18 | 2018 | 23 | Serum | Lublin Voivodeship | 1f | MK381386 MK381440 |
| 211-DK/18 | 2018 | 24 | Lung | Mazovian Voivodeship | 1r | MK381387 MK381441 |
| 213-GK/18 | 2018 | 23 | Serum | Lublin Voivodeship | 1f | – MK381442 |
| 214-MS/18 | 2018 | 25 | Serum | Wielkopska Voivodeship | 1f | MK381388 MK381443 |
| 215-BK/18 | 2018 | 26 | Serum | Swietokrzyskie Voivodeship | 1 g | MK381389 MK381444 |
| 216-JB/18 | 2018 | 27 | Serum | Wielkopska Voivodeship | 1 g | MK381390 MK381445 |
| 217-SM/18 | 2018 | 28 | Serum | Podlaskie Voivodeship | 1e | MK381391 MK381446 |
| 218-KH/18 | 2018 | 29 | Ear notch | Opole Voivodeship | 1r | MK381392 – |
| 219-KH/18 | 2018 | 29 | Ear notch | Opole Voivodeship | 1f | MK381393 – |
| 220-KH/18 | 2018 | 29 | Ear notch | Opole Voivodeship | 1f | MK381394 – |
| 221-KH/18 | 2018 | 29 | Ear notch | Opole Voivodeship | 1f | MK381395 MK381447 |
| 222-KH/18 | 2018 | 29 | Ear notch | Opole Voivodeship | 1r | MK381396 – |
| 223-DA/18 | 2018 | 30 | Ear notch | Wielkopska Voivodeship | 1 g | MK381397 – |
| 224-DA/18 | 2018 | 30 | Ear notch | Wielkopska Voivodeship | 1 g | MK381398 – |
| 225-DA/18 | 2018 | 30 | Ear notch | Wielkopska Voivodeship | 1 g | MK381399 – |
| 226-DA/18 | 2018 | 30 | Ear notch | Wielkopska Voivodeship | 1 g | MK381400 – |
| 228-DA/18 | 2018 | 30 | Ear notch | Wielkopska Voivodeship | 1 g | MK381401 – |
| 232-DA/18 | 2018 | 30 | Ear notch | Wielkopska Voivodeship | 1 g | MK381402 – |

Fig. 3 Distribution of BVDV subtypes in Poland between 2015 and 2018 (percentages)
highest number of isolates (32) and subtypes (4), was identified in Wielkopolskie with the predominance of BVDV-1 g (41%) and BVDV-1b (37%). Second province with the highest number of positive results was Lubelskie, where 8 isolates of BVDV-1f and 5 of BVDV-1b subtypes were found. Only in two provinces (Podlaskie and Lodzkie), where positive results were obtained, single subtypes were identified. Sequence similarity between various subtypes in 5’UTR ranged from 81 to 93%. The identity percentages within same subtypes 1b, 1 g, 1f, 1d, 1r and 1 s were 91.5–100%, 96.5–100%, 91.4–100%, 92.6–100%, 96.5–98%, 99–100% respectively. Sequence similarity between various subtypes in Npro region ranged from 76.5 to 86.5%. The most diverse sequences within the same subtype in Npro region were identified for BVDV-1b with sequence identity values up to 84.9%. The biggest difference in subtype sequences occurred between BVDV-1b and BVDV-1d, while the tiniest variation was observed between BVDV-1f and BVDV-1 s (Fig. 4).

Sequence identity at the amino acid level in Npro region among isolates tested was 78.8–100% and between various subtypes ranged from 78.8 to 93.4%. The biggest differences were observed between BVDV-1d and BVDV-1r and the smallest one between BVDV-1f and BVDV-1 g and also between BVDV-1f and BVDV-1 s. Nucleotide sequences of the BVDV strains have been submitted to GenBank with the following accession numbers: MK044822-MK044827, MK168328-MK168336, MK381356-MK381402 for 5’UTR and MK381419-MK381447 for Npro region.

Discussion

In this study, we investigated the genetic diversity of BVDV isolates from Polish herds collected between 2015 and 2018. PCR amplified sequences were subjected to sequence-based genotyping in 5’ untranslated region. The Npro phylogenetic analysis confirmed typing results obtained for the 5’UTR. Viral isolates were assigned to seven subtypes in descending order of frequency of appearance: 1b, 1 g, 1f, 1d, 1r, 1 s and 1e. Previous study from years 2004–2011 described the circulation of four subtypes of BVDV-1 in Poland (1b, 1d, 1f, 1 g) with predominance of BVDV-1b and BVDV-1d [17]. In later studies, subtype 1e was also detected [18]. Current phylogenetic studies indicate that the number of BVDV subtypes has increased, however BVDV-1b is still the most often detected subtype. It is the most frequently reported subtype of BVDV worldwide. BVDV-1b is predominant in both Americas, Asia and Europe [16]. A large number of isolates belonging to subtype 1f and some of 1 g have been detected in Austria [20] and Italy [21, 22]. BVDV-1f is the most common subtype in Germany and Slovenia [16, 23]. Several studies indicate that 1f and 1 g subtypes may be unique for Europe.

Fig. 4 Matrix of pairwise identity scores generated by alignment of a 371 bp fragment of the Npro gene for 29 Polish isolates and 15 reference strains of BVDV
Viruses of BVD-1 g subtype were isolated more frequently than in the previous study where BVDV-1 g was identified only in two herds [17]. Subtype 1d was predominant in Sweden, in years 2002–2004, when the eradication program was implemented [24]. Strains 183-SY/17, 211-DK/18, 218-KH/18 and 222-KH/18 clustered together with Italian strains belonging to subtype 1r [22]. Three strains (166-KY/15, 167-KY/15, 176-KR/15) form one clade with strains previously identified as 1f (22,146/81) [25] and 1f-like (mousedeer) [26]. Currently, together with the reference strains from Italy [22], they form the 1s subtype [27]. BVDV-1e represented by strain 217-SM/18 has been identified only in one Polish herd. It had 98% nucleotide similarity to the Italian BVDV-1e strain from Northern Italy [28]. This subtype was found also in Switzerland [29] and France [30]. The results of this study show that the genetic heterogeneity of BVDV viruses infecting cattle in Poland has changed. These differences in subtype distribution in comparison to study from years 2004–2011 could be a result of immune selection due to natural infections and also vaccinations, which became very popular in recent years. In the present work, the evidence for geographical clustering of BVDV subtypes was not clear, unlike Italy, where BVDV-1f was predominant in northern Italy while BVDV-1b was the most frequent subtype in southern part of the country [21, 31].

HoBi-like pestiviruses (BVDV-3) do not seem to circulate in Polish cattle and BVDV-2 was found previously only in one herd [19]. BVDV-2 was first identified in North America and was associated with very high mortalities [32] from where the virus was introduced to the European continent [33]. BVDV-2 was also identified in Europe in several countries like: Italy [14], Germany [34] and Austria [20]. So far, natural infections with BVDV-3 in Europe were identified only in Italy [3]. There are suspicions that the virus has been introduced to the European continent through vaccines or other products which were prepared using contaminated bovine serum. The closest genetically related strains to Polish isolates were identified in Slovenia, United Kingdom and Italy according to blastn analysis. High level of similarity among these viruses may suggest a common ancestor. Only a few inactivated and recently also modified-live vaccines are commercially available in Poland. In this study BVDV was identified in 6 herds from animals previously vaccinated with killed vaccines. Three herds were infected with BVDV-1b subtype (strains 187-AN/17, 189-JA/17, 190-JA/17 and 194-TC/17), two with BVDV-1d (184-KN/17, 185-KN/17, 188-LS/17) and one herd was infected with BVDV-1g (215-BK/18). In all these herds protective vaccinations were based on BVDV-1a strain, and they were introduced after PIs removal. Interestingly subtype 1a has never been identified in Poland, which could be the effect of selection force induced by vaccines based on this subtype. Other studies have shown significant differences in antibody levels in serum from calves receiving modified live virus vaccines based on BVDV-2a, with a significantly lower BVDV-1b antibody titres [35]. PI individuals infected with BVDV-1b were identified in one Polish herd vaccinated with a killed vaccine based on BVDV-1a [36]. Although clinical symptoms resembling BVD were not observed in that herd, the protection offered by vaccinal strain did not provide cross protection against BVDV-1b. Vaccination strategy should take into consideration both genetic and antigenic diversity of the virus present in the region where vaccination is implemented and therefore, effective vaccine should include the subtypes of local isolates.

For this reason monitoring of newly emerging strains is important for successful control and eradication programs and it requires constant updates. Antigenic differences among individual subtypes of BVDV-1 occur as well [37]. Therefore, more cross-protection studies should be carried out to address the importance of this diversity. It seems reasonable to include a mixture of several viral subtypes present in local herds when designing effective vaccines. Phylogenetic studies with increasing cattle trade can also help to identify potential sources and routes of virus introduction, although such sources were not identified for Polish isolates, probably due to significant diversity of the virus in every country studied.

The genetic diversity is also important for laboratory diagnosis, since it can hamper the ability of diagnostic methods to identify as many viral subtypes as possible. In this study we used specific primers for non-coding 5’UTR and coding Npro region. 5’UTR is highly conserved among the pestiviruses. It contains cis-acting elements required for viral replication and translation [38]. Npro (N-terminal protein) of BVDV encodes for a cysteine protease that cleaves the N-terminus from the core protein. Npro also prevents interferon-α/β induction in infected cells [39]. The validity of 5’UTR classification in this study was confirmed by the parallel analysis of Npro sequences. RT-PCR used in this study [40], which is commonly used for BVDV detection, does not detect or detects with low efficiency strains of HoBi-like viruses due to the presence of a mismatch at the 3’ end of the forward primer which does not allow proper annealing [41]. This disadvantage may lead to false negative results when testing field samples for BVDV-3 and therefore we implemented real-time PCR enabling the detection of all three species of BVDV with high sensitivity. This new method was implemented to study doubtful PCR results although all samples turned negative when tested with real-time PCR.
| Pestivirus species | Subtype | Strain   | 5’UTR Accession number | NPro Accession number |
|-------------------|---------|----------|------------------------|-----------------------|
| BVDV-1            | 1a      | NADL     | AJ133738               | AJ133738              |
| BVDV-1            | 1a      | Singer   | DQ088995               | DQ088995              |
| BVDV-1            | 1b      | VEDEVAC  | AJ585412               | AJ585412              |
| BVDV-1            | 1b      | OSLOSS   | AY279528               | M96687                |
| BVDV-1            | 1b      | Manas-1  | EU555288               | –                     |
| BVDV-1            | 1b      | New York-1 (NY-1) | FJ387232 | FJ387232 |
| BVDV-1            | 1b      | KE9      | EF101530               | EF101530              |
| BVDV-1            | 1c      | Shitara/01/05 | AB359926 | AB359926 |
| BVDV-1            | 1c      | GS1      | –                      | JQ071526              |
| BVDV-1            | 1c      | Letuyi   | EU159701               | –                     |
| BVDV-1            | 1c      | Manasi   | EU159702               | –                     |
| BVDV-1            | 1d      | F        | AF298065               | AF287284              |
| BVDV-1            | 1d      | OK1(CAJ)NCP/03 | AB359927 | AB359927 |
| BVDV-1            | 1d      | Duland44 | –                      | KC414609              |
| BVDV-1            | 1d      | 10JJ-SKR | KC757383               | KC757383              |
| BVDV-1            | 1d      | BJ1308   | –                      | KT951841              |
| BVDV-1            | 1e      | SLO/2407/2006 | KX577637 | KX577637 |
| BVDV-1            | 1e      | CN11a@09 | MG434588               | –                     |
| BVDV-1            | 1e      | CH-05-02 | –                      | EU180036              |
| BVDV-1            | 1f      | J-AT     | FJ493480               | –                     |
| BVDV-1            | 1f      | J        | –                      | AF287286              |
| BVDV-1            | 1f      | W        | –                      | AF287290              |
| BVDV-1            | 1f      | O-1897/00-175 | AY323895 | AY323895 |
| BVDV-1            | 1f      | G-1703/99-43 | AY323876 | AY323876 |
| BVDV-1            | 1f      | E-1411/00-9 | AY323872 | –             |
| BVDV-1            | 1f      | B99/05   | –                      | EU224259              |
| BVDV-1            | 1g      | L        | FJ493483               | AF287287              |
| BVDV-1            | 1g      | A-AT     | FJ493482               | –                     |
| BVDV-1            | 1g      | A        | AF298064               | AF287283              |
| BVDV-1            | 1g      | 10/08    | JN715004               | –                     |
| BVDV-1            | 1g      | 48/08    | –                      | JNB33739              |
| BVDV-1            | 1h      | G        | AF298066               | AF287285              |
| BVDV-1            | 1h      | CH6569   | MH907191               | –                     |
| BVDV-1            | 1h      | B80/05   | EU224239               | –                     |
| BVDV-1            | 1h      | CH-95-11 | –                      | EU180042              |
| BVDV-1            | 1i      | 23–15    | AF298059               | AF287279              |
| BVDV-1            | 1i      | 2186     | –                      | JQ920329              |
| BVDV-1            | 1i      | MR02497  | LT902628               | –                     |
| BVDV-1            | 1j      | KS86-1ncp | AB078950 | AB078950 |
| BVDV-1            | 1j      | 2/Vr/95  | AJ293594               | –                     |
| BVDV-1            | 1j      | Deer-G81 | –                      | U80902                |
| BVDV-1            | 1k      | SuwaCp   | AF117699               | AY794998              |
| BVDV-1            | 1k      | CH7247   | MH907869               | –                     |
| BVDV-1            | 1k      | Bohni    | –                      | AY949997              |
In summary, the distribution of subtypes in Poland has changed. Two new subtypes 1r and 1s were detected for the first time. Monitoring of strains circulating in a given country is a useful indicator in the aspect of designing an effective vaccination program or a reliable diagnostic test.

### Table 2: List of reference strains used for phylogenetic comparison with Polish isolates (Continued)

| Pestivirus species | Subtype | Strain | 5’UTR Accession number | NPro Accession number |
|--------------------|---------|--------|------------------------|-----------------------|
| BVDV-1             | 11      | 71-03  | KF205294               |                       |
| BVDV-1             | 11      | 71-15  | KF205306               | KF205329              |
| BVDV-1             | 11      | CH-01-08 | –                  | EU180033              |
| BVDV-1             | 1m      | LZ05   | GU120241               |                       |
| BVDV-1             | 1m      | ZM-95  | AF526381               | AF526381              |
| BVDV-1             | 1m      | XC     | –                      | MH166806              |
| BVDV-1             | 1n      | Shitara/02/06 | AB359930          | AB359930              |
| BVDV-1             | 1n      | So CP/75 | AB359929            | AB359929              |
| BVDV-1             | 1o      | AQGN96815 | AB300691            |                       |
| BVDV-1             | 1o      | IS25CP/01 | AB359931            | AB359931              |
| BVDV-1             | 1o      | HA2-12 | –                      | KX218370              |
| BVDV-1             | 1p      | BJ0701 | GU120247               | GU120259              |
| BVDV-1             | 1p      | BJ0702 | GU120248               | GU120260              |
| BVDV-1             | 1q      | camel-6 | KC695810             | KC695810              |
| BVDV-1             | 1q      | SD0803 | JQ400273               | JN400273              |
| BVDV-1             | 1r      | VE/245/12 | LM994671           |                        |
| BVDV-1             | 1r      | CA/181/10 | LM994672           | –                      |
| BVDV-1             | 1r      | 79/11  | KY040384               | KX218370              |
| BVDV-1             | 1r      | 103/11 | KY040372               | KY040425              |
| BVDV-1             | 1s      | UW/136/08 | LM994673            | LN515612              |
| BVDV-1             | 1s      | mousedeer | AY158154          | –                      |
| BVDV-1             | 1s      | 2561   | JQ920287               | JQ920343              |
| BVDV-1             | 1s      | 22,146/81 | AJ304376           | –                      |
| BVDV-1             | 1t      | SI/207/12 | LM994674          | LN515611              |
| BVDV-1             | 1u      | M31182 | JQ929141               | JQ799141              |
| BVDV-2             | 2a      | New York/93 | AF502399         | KR093034              |
| BVDV-2             | 2a      | 890    | L32886                 | –                      |
| BVDV-2             | 2a      | J205-1 | GQ888686               | GQ888686              |
| BVDV-2             | 2b      | Soldan | U94914                 | AY735495              |
| BVDV-2             | 2b      | Giessen 6 | AY379547          | –                      |
| BVDV-2             | 2b      | Hokudai-Lab/09 | –                 | AB567658              |
| BVDV-2             | 2b      | LV60-57-13 | –                  | KM217405              |
| BVDV-2             | 2c      | NRW 12-13 | HG426483           | HG426483              |
| BVDV-3             | 3       | Th/04_KhonKaen (TKK) | FJ040215       | FJ040215              |
| BVDV-3             | 3       | Italy-83/10-cp | JQ612705       | JQ612705              |
| BDV                | –       | X818   | AF037405               | AF037405              |
| CSFV               | –       | Alfort/187 | NC038912         | NC038912              |

### Conclusion
In summary, the distribution of subtypes in Poland has changed. Two new subtypes 1r and 1s were detected for the first time. Monitoring of strains circulating in a given country is a useful indicator in the aspect of designing an effective vaccination program or a reliable diagnostic test.

### Methods
#### Sample collection
A total of 9290 serum, tissue homogenate, ear notch and semen samples were collected in years 2015–2018. The animals used in the study came from private farms, where infection with BVDV was suspected based on
clinical symptoms or where eradication was under way. The owners of those herds provided local vets with their permissions to collect samples for laboratory testing. Samples were collected in 8 out of 16 provinces of Poland: Kujawsko-Pomorskie, Lubelskie, Łódzkie, Opolskie, Świętokrzyskie, Mazowieckie, Wielkopolskie and Podlaskie. Cattle population in last three provinces comprises 51% of the total population of this ruminant species in Poland. For comparison studies sequences of 81 reference strains of different species and subtypes of BVDV and single strains of BDV and CSFV were retrieved from GenBank (Table 2).

RNA extraction and RT-PCR
Total RNA was extracted using TRI Reagent (Sigma-Aldrich, USA) from 500 μl of serum, tissue homogenates, cell culture medium after overnight soaking of ear notchers or from diluted semen following the manufacturer’s instructions and stored at -80 °C until testing. Reverse transcription-polymerase chain reaction (RT-PCR) was carried out using the Transcriptor One-Step RT-PCR Kit (Roche) in a 25 μl reaction mix consisting of PCR buffer 5 μl, water DEPC 15.5 μl, set of primers 1 μl (10 μM), 0.5 μl enzyme mix and 2 μl of template RNA. Reverse transcription was performed at 50 °C for 30 min using reverse primer. cDNA was amplified using primers pair specific for BVDV 5′ untranslated region: 324F (5′-ATGCCCTWTAGTAGGACTAGCA-3′) and 326R (5′-TCAACTCCATGTGCCATGTAC-3′) [40]. PCR thermal conditions were the following: initial denaturation at 94 °C for 7 min followed by 35 cycles of denaturation at 94 °C for 10 s, primer annealing at 53 °C for 30 s and elongation at 68 °C for 30 s. The final elongation was extended to 7 min at 68 °C. Primers specific for Npro region: B32-F (TGCTACTAAAAATCTCTGCTGT) and B31-R (CCATCTATrCAYACATArATGTGGT) [23] were used with thermal profile of 94 °C for 15 s, 50 °C for 30 s and 68 °C for 1 min for 35 cycles and 10 min in 68 °C for final elongation. Approximate sizes of PCR products were 288 bp and 441 bp for 5′UTR and Npro region respectively.

Sequencing and phylogenetic analysis
The PCR products were sequenced in both directions with the same primers used for amplification using Big Dye Terminator v3.1 Cycle Sequencing Kit with a 3730XL Genetic Analyzer (Applied Biosystems). The DNA fragments were purified using a QIAquick PCR Purification kit (Qiagen), following the analysis in a 16-capillary sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The consensus of each genetic region was determined by the alignment of forward and reverse strand sequences using Clustal Omega tool of the European Molecular Biology Laboratory (http://www.ebi.ac.uk). Sequences generated in this study were aligned with the analogous sequences of reference pestivirus strains deposited in the GenBank database (Table 2) using the ClustalW algorithm from Molecular Evolutionary Genetics Analysis software package, version 5.2 (MEGA 5.2). Phylogenetic trees were constructed using neighbor-joining algorithm [42] with a Kimura 2-parameter substitution model [43] with 1000 bootstrap replicates. Phylogenetic trees were also constructed by the Bayesian method with the GTR substitution model using the tree-builder tool of the Geneious software [44]. Sequence identity (%) among strains was calculated using the identity matrix in BioEdit v.7.2.5 software [45].

Additional files

**Additional file 1**: Phylogenetic relationship between field and reference strains inferred by Bayesian analysis in 5′UTR. The figure shows a phylogenetic tree created on the basis of the 5′UTR fragment by the Bayes method with the GTR substitution model. It consists of 62 field isolates and representatives of all known subtypes of the BVDV-1 species, representatives of the BVDV-2, BDV and CSFV species. (PDF 148 kb)

**Additional file 2**: Phylogenetic relationship between field and reference strains inferred by Bayesian analysis in Npro region. The figure shows a phylogenetic tree created on the basis of the fragment of the Npro region by the Bayes method with the GTR substitution model. It consists of 29 field isolates and representatives of all known subtypes of the BVDV-1 species, representatives of the BVDV-2, BDV and CSFV species. (PDF 120 kb)

**Abbreviations**
- S′UTR: Untranslated region S′; BVDV: Bovine viral diarrhea virus; Npro: N-terminal protease; PCR: Polymerase chain reaction; PI: persistently infected; RT: Reverse transcription

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**Authors’ contributions**
MP supervised the project. 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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**Availability of data and materials**
The data sets supporting the results of this article are included within the article.

**Ethics approval and consent to participate**
The material used in this study consisted of field samples collected during clinical examination of animals and these animals were not used for experimental studies. Tissue samples were collected from dead animals by local vets after verbal approvals from the owners for further testing. The approval from ethics committee was not required according to national
regulation (“Act on the Protection of Animals Used for Scientific or Educational Purposes” published in the Journal of Laws of 2015, item 266 from 15 January, 2015).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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