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Use of multivariate analysis to evaluate antigenic relationships between US BVDV vaccine strains and non-US genetically divergent isolates

Ana Cristina S. Mosena [a,1], Shollie M. Falkenberg [a,*], Hao Ma [a], Eduardo Casas [a], Rohana P. Dassanayake [a], Richard Booth [b], Gian Mario De Mia [c], Matthias Schweizer [d], Cláudio W. Canal [e], John D. Neill [a]

[a] Ruminant Disease and Immunology Research Unit, National Animal Disease Center, USDA, Agricultural Research Service, Ames, IA, United States
[b] Pathobiology and Population Sciences, Royal Veterinary College, Hawkshead Lane, Hatfield, Hertfordshire, AL9 7TA, United Kingdom
[c] Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche "Togo Rosati", Perugia, Italy
[d] Institute of Virology and Immunology, Department of Infectious Diseases and Pathobiology, Vetsuisse Faculty, University of Bern, Bern, Switzerland
[e] Laboratório de Virologia Veterinária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil

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ABSTRACT

Bovine viral diarrhea virus (BVDV) comprises two species, BVDV-1 and BVDV-2. But given the genetic diversity among pestiviruses, at least 22 subgenotypes are described for BVDV-1 and 3-4 for BVDV-2. Genetic characterization is generally accomplished through complete or partial sequencing and phylogeny, but it is not a reliable method to define antigenic relationships. The traditional method for evaluating antigenic relationships between pestivirus isolates is the virus neutralization (VN) assay, but interpretation of the data to define antigenic relatedness can be difficult to discern for BVDV isolates within the same BVDV species. Data from this study utilized a multivariate analysis for visualization of VN results to analyze the antigenic relationships between US vaccine strains and field isolates from Switzerland, Italy, Brazil, and the UK. Polyclonal sera were generated against six BVDV strains currently contained in vaccine formulations, and each serum was used in VNs to measure the titers against seven vaccine strains (including the six homologous strains) and 23 BVDV field isolates. Principal component analysis (PCA) was performed using VN titers, and results were interpreted from PCA clustering within the PCA dendrogram and scatter plot. The results demonstrated clustering patterns among various isolates suggesting antigenic relatedness. As expected, the BVDV-1 and BVDV-2 isolates did not cluster together and had the greatest spatial distribution. Notably, a number of clusters representing antigenically related BVDV-1 subgroups contain isolates of different subgenotypes. The multivariate analysis may be a method to better characterize antigenic relationships among BVDV isolates that belong to the same BVDV species and do not have distinct antigenic differences. This might be an invaluable tool to ameliorate the composition of current vaccines, which might well be important for the success of any BVDV control program that includes vaccination in its scheme.

1. Introduction

Three out of 11 currently accepted viral species from the genus Pestivirus, within the family Flaviviridae, exist in most cattle-producing countries worldwide (Yeşilbağ et al., 2017). The BVDV-1, BVDV-2 and HoBi-like pestivirus (HoBiPeV) species represent important economic impact for the cattle industry, mainly due to reproductive losses on all pregnancy stages and development of persistently infected (PI) calves upon in utero infection. Such PI animals mount no immune response against the infecting virus and constantly secrete high amounts of infectious virus particles and maintain the infection cycle within the herd (Richter et al., 2017; Pinior et al., 2019; Rodning et al., 2012; Basqueira et al., 2020).

Pestiviruses can be classified into two biotypes, noncytopathic (ncp)

* Corresponding author at: Ruminant Disease and Immunology Research Unit, National Animal Disease Center, USDA, Agricultural Research Service, 1920 Dayton Ave, Ames, IA, 50010, United States.
E-mail address: shollie.falkenberg@usda.gov (S.M. Falkenberg).
[1] Permanent address: Laboratório de Virologia Veterinária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil.

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and cytopathic (cp), according to their activity in cell culture, where cp viruses kill infected cells and ncp viruses do not. In addition, pestivirus species (Smith et al., 2017) possess a high genetic diversity due to several mechanisms, such as high rate of mutations during replication, a feature common to RNA viruses (Becher and Tautz, 2011; Jones and Weber, 2004). The broad genetic diversity within species, especially BVDV-1 and 2, led to the necessity of a further classification of isolates into subgenotypes. Based on phylogenetic analysis of genomic regions, BVDV-1 can be currently classified into at least 22 subgenotypes (1a to 1 u) and BVDV-2 into 3–4 subgenotypes (2a to 2c, and potentially 2d). The most prevalent subgenotypes worldwide are 1a, 1b and 2a, and most of the less prevalent subgenotypes are restricted to European, Asian and South American countries (Yeşilbağ et al., 2017; Silveira et al., 2017).

Several in vitro studies have attempted to link genetic to antigenic characterization, in an attempt to establish if isolates of the same subgenotype would present similar antigenic characteristics, but divergent results were found (Minami et al., 2011; Ridpath et al., 2010; Pecora et al., 2014). Antigenic differences not only between species (Bauermann et al., 2012; Ridpath et al., 1994), but also between subgenotypes and even between isolates of the same subgenotype were described (Minami et al., 2011; Ridpath et al., 2010; Ridpath, 2003; Bachofen et al., 2008). However, the practical consequences of the antigenic diversity between subgenotypes are still not know (Ridpath et al., 1994; Bachofen et al., 2008; Ridpath, 2005; Sozzi et al., 2020a). While the clear difference in levels of cross-neutralization between BVDV-1, 2 and HoBiPeV neutralizing antibodies have been widely described, identifying antigenically divergent or similar isolates within the same species and subgenotype by the use of VN assay can be challenging. The broad diversity of isolates, varying levels of cross-reactivity between multiple isolates and interpretation of a great amount of data are barriers to a clear interpretation of VN assay results. Typically, serological relatedness is expressed as coefficient of antigenic similarity (R) between strains using VN titers. There are a limited number of studies that have evaluated the serological relationships that exist among isolates from different BVDV subgenotypes (Minami et al., 2011; Ridpath et al., 2010; Bachofen et al., 2008; Becher et al., 2003; Couvreur et al., 2002) and these studies have aimed at evaluating the antigenic variability not only between BVDV species but within BVDV subgenotypes. Recently, a multivariate analysis called Principal Component Analysis (PCA) was for the first time applied to BVDV antigenic characterization (Mosena et al., 2020), and antigenic clusters and differences could be identified. This contrasts with previous reports (Couvreur et al., 2002) where BVDV-1 isolates were homogeneous since BVDV-1 isolates could not be subdivided into smaller serotypes based on neutralization patterns and R value calculations. While smaller serological subgroups could not be determined, some of the VN titers among BVDV-1 strains and antisera were rather low (>20) and these VN titers were as low as the VN titers observed between BVDV-1 and BVDV-2 strains and their corresponding antisera (Couvreur et al., 2002).

Given that most current commercially available BVDV vaccines contain BVDV-1a and/or 2a strains, there is concern about the level of cross protection that can be attained against other BVDV species and subgenotypes. Although, studies have demonstrated that BVDV-1a vaccinated cattle were proven to be fully or in part protected against BVDV-1b infection (Brock and Cortese, 2001; Xue et al., 2010). While these vaccination/challenge studies support the thinking that current vaccines confer protection against other subgenotypes, PI surveillance studies would suggest a lack of protection as the most frequent subgenotype detected in BVDV PI calves in US is BVDV-1b (Fulton et al., 2009; Workman et al., 2016). In addition, recently other genetically diverse isolates (1c, 1i, 2b and 2c subgenotypes) were recently identified for the first time (Neill et al., 2019a; Neill et al., 2019b) in the US, increasing the genetic diversity of BVDV in this country, raising more concerns about the level of cross protection associated with current vaccines. Given the increased genetic diversity within the US and genetic diversity that is observed globally, a better understanding is necessary to determine the level of protection that can be attained with current BVDV vaccines against other pestivirus species and subgenotypes. This information is imperative with regard to development of new vaccines or vaccination strategies that can elicit great cross protection against genetically and antigenically diverse isolates. Historical data would suggest that the breadth of the genetic and antigenic diversity will continue to increase. Therefore, a better understanding of antigenic relationships is required to inform potential intervention strategies such as vaccination as part of control programs moving forward.

The aim of this study was to use the PCA approach for interpretation of VN data from genetically distinct BVDV-1 and 2 field isolates from several subgenotypes and distinct geographical origins, and to describe the cross neutralizing patterns of serum raised against 1a and 2a strains used in current vaccine formulations in neutralizing these genetically divergent BVDV field isolates.

2. Material and methods

2.1. Viruses

Twenty-three field isolates, twenty-two field isolates representing BVDV-1 subgenotypes (1a, d, e, f, g, h, i and k) along with one HoBiPeV isolate (HoBi/D32), and seven US based vaccine strains (3 BVDV-1a and 4 BVDV-2a) were selected for this study (Table 1). The field isolates were selected in an attempt to represent a broad genetic diversity within the BVDV-1 species in contrast to strains used in most BVDV vaccines, which are 1a and 2a strains of US origin. In the USA, the majority of field isolates are classified as 1a, 1b and BVDV-2a, with few descriptions of BVDV-2b and recent descriptions of BVDV-1c, BVDV-1i and BVDV-2c subgenotypes (Neill et al., 2019a; Neill et al., 2019b). Non-US isolates provided greater genetic diversity, as selection of non-US field samples for antigenic characterization included 22 cp and ncp BVDV-1 field isolates from Italy (1e, 1f, 1g and 1k subgenotypes), Switzerland (1e, 1h and 1k), United Kingdom (1a, 1d, 1e and 1i), and one HoBi-like pestivirus isolate from Brazil (Table 1). The seven BVDV vaccine strains (3 BVDV-1a and 4 BVDV-2a) chosen for the current study are routinely used in BVDV modified-live vaccine formulations and also in killed vaccines, although a couple of strains contained in some killed vaccines were not represented in this study. Six out of the seven vaccine strains were used to generate antiserum that was subsequently used in VN assays.

The seven vaccine strains and 23 field isolates were propagated in Madin-Darby bovine kidney cells (MDBK) the laboratory according to standard protocol. Each strains and isolate aliquot was used to inoculated a flask of MDBK cells with 75 % confluence layer for 1 h, followed by cell layer wash to remove the inoculum and addition of fresh Dulbecco's Modified Eagle's Medium (DMEM) containing 10 % of fetal bovine serum (FBS) for incubation at 37 °C in a 5% CO2 atmosphere for 4–5 days. Cytopathic effect was observed in MDBK cells inoculated with cp strains and field isolates. Viruses were titered through serial 10-fold dilutions with replicates of five wells per dilution in bovine turbinate (BT) cells. Cytopathic effect was observed in wells inoculated with cp strains and field isolates, and for ncp isolates the cell layer was fixed and stained according to standard immunoperoxidase staining protocol using the E2 protein-specific monoclonal antibody N2 and horseradish peroxidase-conjugated protein G (Bolin and Ridpath, 1995). Titration 96-well plates were briefly rinsed with distilled water and cells fixed in 60/40 PBS-BSA/acetone at room temperature and dried for 1 h at 37C°. Cells were then incubated with E2 protein-specific monoclonal antibody N2 diluted in PBST for 1 h. After application of this primary antibody, wells were rinsed twice with PBS wash buffer in between steps. Cell layers were then incubated with 1:60 dilution of goat antiserum to mouse gamma globulin (IgG) (Cappel, catalog number 55455) in PBSTN binding buffer for 1 h. Cells were then incubated with rec-Protein G-Peroxidase Conjugate (ZYMED, catalog number 10-1223) diluted in

Table 1
Strains and isolates used in this study with referred ID, genetic classification, country of origin and GenBank accession number.

	Country	Species/ Subgenotype	Isolate ID / Virus ID	Complete Genome Genbank Acession no.	Biotype
Field isolates	Italy	1e	D7219_1e / MA/101/05	MW054940	ncp
		1f	D14688 _1f / LA/230/14	MW054933	cp
			D18648_1f / LA/87,88,90/05	MW054934	ncp
		1 g	D23284_1 g / UM/111/06	MW054936	ncp
		1k	D18892_1k / TO/197/11	MW054935	ncp
			D59460_1k / SA/159/09	MW054937	ncp
	Swiss	1e	CH_Maria_1e	MW655625	ncp
			S03_1175_1e	MW655631	ncp
			Carlito_1e	KP313732	ncp
			R2000_95_1e	MW655627	ncp
		1 h	CH_04_01b_1 h	MW655625	ncp
			R3572/90_1 h	MW655629	ncp
			SM09_20_1 h	MW655632	ncp
		1k	CH_Suwa_1k	AY894998	ncp
			R3230/95_1k	MW655628	ncp
			R5013/96_1k	MW655630	ncp
	UK	1a	62_2_1a	MW250798	ncp
			63_1_1a	MW250799	ncp
		1d	67_1_1d	MW250800	ncp
		1e	68_1_1e	MW250802	ncp
		1i	58_2_1i	MW250797	ncp
			69_1_1i	MW250803	ncp
	Brazil	HoBiPeV	HoBi/D32	AB871953	ncp
Vaccine strains	USA	1a	C24V _1a	AF091605	cp
			Singer _1a	DQ088995	cp
			NADL _1a	M31182	cp
		2a	125c_2a	MH806434	cp
			296c_2a	MH806436	cp
			5912c_2a	MH231129	cp
	Canada	2a	53637c_2a	MH231127	cp

PBSTN binding buffer for 1 h at room temperature. Staining was developed with AEC substrate prepared in acetate buffer with hydrogen peroxide until red color appeared and wells were rinse with tap water. All cell lines, medium and fetal bovine serum were tested free of pestivirus antigen and antibodies (Ridpath et al., 1994).

2.2. Genetic characterization

All isolates and vaccine strains used in this study are available in GenBank and specific details can be found in Table 1. Isolates previously described but that had no 5′UTR or E2 sequence available at GenBank (all European field isolates with exception of Carlito_1e (Stalder et al., 2015) and CH_Suwa_1k (Bachofen et al., 2008)) had the whole genome sequenced according to previously described protocol (Neill et al., 2019b) and submitted to Genbank (Table 1). Simultaneous multiple whole genomes sequencing was obtained through cDNA synthesis of viral RNA with primers composed of 20 bases of known sequence with 8 random bases at the 3′-end, so that resultant cDNAs could be barcode identified and amplified by primer-specific PCR and sequenced on the Ion Torrent PGM platform. Virus genomes were assembled by both de novo and reference-assisted assembly methods. Nevertheless, phylogenetic analysis for subgenotype classification was performed using the 5′UTR sequences of all isolates and strains, as 5′UTR phylogeny is commonly accepted and used for subgenotype classification (Bauermann et al. (2012); Ridpath et al. (1994)). All viruses were confirmed to be classified as same subgenotype as literature. A more detailed phylogenetic analysis of the E2 amino acid sequence of the same strains and isolates was obtained so the genetic relationship between viruses could be compared to the antigenic characterization. A neutralizing humoral immune response in pestiviruses infections is mostly directed against the highly immunogenic E2 protein. To determine amino acid differences in the major neutralizing glycoprotein E2 (Fulton et al., 1995; Deregt et al., 1998), MEGA6 software tools Clustal W alignment and UPGMA method were used to obtain the phylogenetic tree with branch support estimated using 1000 bootstrap replicates and Poisson correction method used to calculate evolutionary distances.

2.3. Antisera

From the seven selected vaccine strains, specific antiserum was generated against six strains (C24V_1a, Singer_1a, NADL_1a, 125_2a, 296_2a and 53637_2a). The same antisera were used in a previous study where PCA was applied in VN assay results interpretation (Mosena et al., 2020). Briefly, antisera were generated by intranasal instillation (2.5 ML/nostril, 1 × 106 TCID50/ML) of each viral preparation in BVDV antigen/antibody-free colostrum deprived calves according to

previously described protocol (Neill et al., 2019a). Each calf received a subcutaneous booster injection of 2 ML of virus after 21–28 days, and at approximately 48 days a sample of blood was drawn by jugular puncture and serum was prepared and stored at −20C for use in VN assays. All animals were handled in accordance with the Animal Welfare Act Amendments (7 U.S. Code §2131 to §2156) and all study procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the National Animal Disease Center (protocol #ARS-2017–673). The vaccine strain 5912_2a was not included in the antiserum production.

2.4. VN assays

VN assays were performed according to previously described BVDV VN protocol (Bolin and Ridpath, 1995) and as previously described utilizing the PCA analysis (Mosena et al., 2020), where each of the six antisera were tested against the seven vaccine strains (six of them used as inoculum for the antisera production, called homologous strains) and the 23 genetically divergent field isolates from different subgenotypes and geographic origin (Table 1). In 96-well plates, 50 u L of serial two-fold dilutions of one antiserum was incubated with 50 TCID50 of virus per well (replicates of five wells per dilution)for one hour, and then 2 × 10^4 bovine turbinate cells were inoculated per well. Four days after inoculation, cp effect was observed on each well for cp strains, while growth of ncp viruses was tested using monoclonal antibody N2 (E2 protein-specific) and horseradish peroxidase-conjugated protein G as described in Viruses methods section and according to previously described (Bolin et al., 1991). Neutralization titers of each antiserum against each of the strains and isolates were calculated using the Spearman-Kärber method (Sozzi et al., 2020b). The same methodology was performed for each of the six antisera against the 30 strains and isolates.

2.5. VN titers distribution and PCA

The VN titers were calculated, transformed into log2 values and used to generate distribution of the data represented by box and whisker plots. These values were then used to conduct PCA using the prcomp function in R. The first and second principle component (PC) were then used to generate the cluster analysis dendrogram by hierarchical cluster analysis with an unweighted pair group mean arithmetic (UPGMA) method using the function hclust in R. The relative positions of the subgenotypes were drawn by ggplot with the first PC (PC1) representing the x and second PC (PC2) representing the y axis. All the analyses were conducted by R (version 3.6.1).

3. Results

3.1. E2 amino acid phylogeny

The phylogenetic tree generated with E2 amino acid sequences of the 30 strains and isolates showed that the HoBi/D32 isolate was the most divergent of the ruminant pestiviruses, presenting a low amino acid identity to BVDV-1 and 2 species. Within the BVDV species branch, two main clusters grouped all BVDV-1 and 2 strains and isolates according to the previous classification described in literature (Fig. 1). The criteria for clusters analysis was established as 9 amino acids substitution per 100 residues (Fig. 1), in order to keep vaccine strains classified as 1a subgenotype in the same cluster, since these strains have been extensively characterized for many years.

In the BVDV-1 branch, 1a vaccine strains were located in two different clades. One of the clades included the strain C24V_1a and two UK 1a field isolates, and the other clade contained the strains NADL_1a and Singer_1a (Fig. 1). 1i isolates (58_2_1i and 69_1_1i) were clustered together, while isolates belonging to the 1e subgenotype were separated into two clusters, where two Swiss 1e isolates generated one cluster (CH_Maria_1e and Carlito_1e) and the other Swiss isolates along with UK isolate 68_1_1 and Italian D7219_1e formed an individual cluster (Fig. 1). One clade included all 1k isolates, and the same happened to 1 h and 1f clade. Subgenotypes that were represented by one isolate (67_1_1d and D23284_1 g) generated single isolate clusters (Fig. 1).

The BVDV-2 vaccine strains clustered into one clade, consisting of one clade with 296_2a, 5912_2a and 125_2a strains grouped together while vaccine strain 53637c_2a was divergent from others and was placed in a separated branch (Fig. 1).

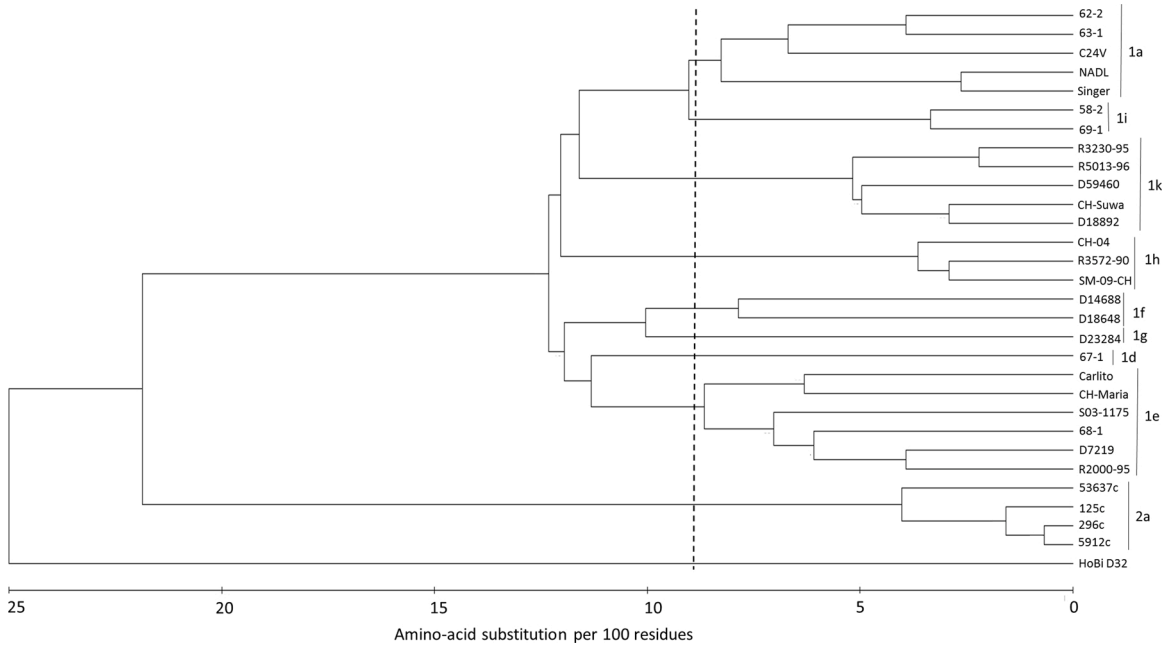

Fig. 1. Phylogenetic analysis of the E2 coding sequence of BVDV. Phylogenetic analysis of the E2 sequences of 30 BVDV strains and isolates (5 BVDV-1a, 1 BVDV-1d, 6 BVDV-1e, 2 BVDV-1f, 1 BVDV-1g, 3 BVDV-1h, 2 BVDV-1i, 5 BVDV-1k, 4 BVDV-2a, and 1 HoBi) to determine amino acid differences within the E2 protein among strains for the major neutralizing protein.

3.2. VN titers distribution

The distribution of VN titers for the six antisera against the BVDV isolates (Supplementary Table 1) were plotted using box and whisker plot (Fig. 2). VN titers (log2) were distributed along the X axis for each antiserum in Y axis. In Fig. 2, the mean was denoted by bar, median titers were denoted by a dot, and boxes extended from the 25th to the 75th percentile with whiskers indicating the minimum and maximum titer (Fig. 2). The maximum titer in all antisera was against the strain which the antisera was specifically generated. Antisera generated against Singer_1a and 53637_2a had the highest mean neutralizing titers and, in a comparison of the means of the neutralizing titers, antisera against BVDV-1 vaccine strains had the highest mean titers (Fig. 2A), as among the isolates there were no BVDV-2 isolates.

3.3. Antigenic clusters through PCA

As previously described (Mosena et al., 2020), results from the PCA yielded two different illustrations of the data, both a PCA cluster dendrogram (Fig. 3A-5A) and scatter plot (Fig. 3B-5B). The PCA cluster dendrogram combines the variation from both PC1 and PC2 into one value to cluster the strains and isolates into antigenically similar groups as determined by VN titers and this is accomplished by hierarchical cluster analysis with an unweighted pair group mean arithmetic (UPGMA) method using the function hclust in R.Whereas the two-dimensional PCA scatter plot represents two axes, the PC1 associated with the X axis and PC2 associated with the Y axis, and the relative positions of the subgenotypes were drawn by ggplot in R. The percent variability represented by PC1 and PC2 is denoted on each axis. These plots allowed the identification of viruses that cluster together in the PCA dendrogram and subsequent categorization of these isolates to groups within the PCA scatter plot. Collectively, by superimposing the PCA cluster dendrogram groups on to the scatter plots the spatial orientation of isolates and potential antigenic groups could be assessed. The antigenic groups are denoted by Roman numerals (I, II, III, etc.) and color shading was utilized to better characterize antigenic groups within each respective Figure. A height of 1 within the PCA cluster dendrogram was used as the minimum cutoff value to characterize strains and isolates that cluster together into the antigenic groups based on VN results and this criterion was used to superimposed on the PCA scatter plot.

The dataset (Figs. 3, 4 and 5) was analyzed using both BVDV-1a and BVDV-2a antisera titers (Fig. 3), only BVDV-1a antisera titers (Fig. 4) and only BVDV-2a antisera titers (Fig. 5). A height of 1 within the PCA cluster dendrogram was used as the criteria to characterize strains and isolates into like groups (Figs. 3, 4 and 5A). The PCA two-dimensional approach generated a scatter plot where the contribution of each PC value can be visualized. When viruses that clustered into like groups in the dendrogram were identified within the PCA scatter plot, the spatial orientation of antigenically related groups could be visualized, as the PC1 and PC2 contribution to the variability of the data (Figs. 3, 4 and 5B).

To identify groups with similar neutralization patterns, initial antigenic comparisons using the PCA were obtained by evaluating VN titers using the six vaccine strains antisera against a total of 30 viruses presented here that included 7 vaccine strains and 23 non-US origins genetically divergent BVDV field isolates belonging to subgenotypes not typically observed in the US (Fig. 3). Two main branches can be observed in the PCA cluster dendrogram, with BVDV-1 and -2 viruses forming the two major branches. Five clusters formed by more than one virus were identified within the BVDV-1 main cluster. Singer_1a, NADL_1a, and C24V_1a vaccine strains formed one cluster (VI), but no field isolates clustered with the vaccine strains (Fig. 3A). The remaining four clusters within the BVDV-1 main cluster were comprised of field isolates apart from Italian D23284_1 g and D14688_1f isolates forming individual branches (Fig. 3A). Cluster V only contained BVDV-1i isolates (Fig. 3A). Most BVDV-2 strains and isolates were grouped into one cluster (I), while viruses 5912_2a and HoBi/D32 formed individual branches within the BVDV-2 main cluster (Fig. 3A).

The PCA scatter plot generated a two-dimensional view of the plot and demonstrated the spatial position of the strains and isolates in relation to each other, as well as the position in the PC1 and PC2 axis (Fig. 3B). The PC1 represented by the X axis in the PCA scatter plot was representative of 73.07 % of the variability (Fig. 3B). While a different representation of the data, the two main branches observed in the PCA dendrogram illustrate similar categorization as the PC1 axis largely grouped isolates into BVDV-1 and BVDV-2 species. Thus, BVDV-2 strains were plotted in the left PC1 quadrant, very distant from BVDV-1 groups in the right quadrant (Fig. 3B). Isolates that formed individual clusters in the PCA cluster dendrogram (Fig. 3A) were observed in the scatter plot as having no proximity to the multiple isolates/strains groups (Fig. 3B). In addition, PC2 represented by the Y axis accounted for 18.07 % of the variability (Fig. 3B). For the cluster of viruses containing the vaccine strains, cluster I for the BVDV-2 strains and cluster VI for the BVDV-1 strains, the VN titers are higher than the other cluster of viruses, which would be expected given that the homologous titers, in general, are greater than heterologous titers among other viruses. This is best

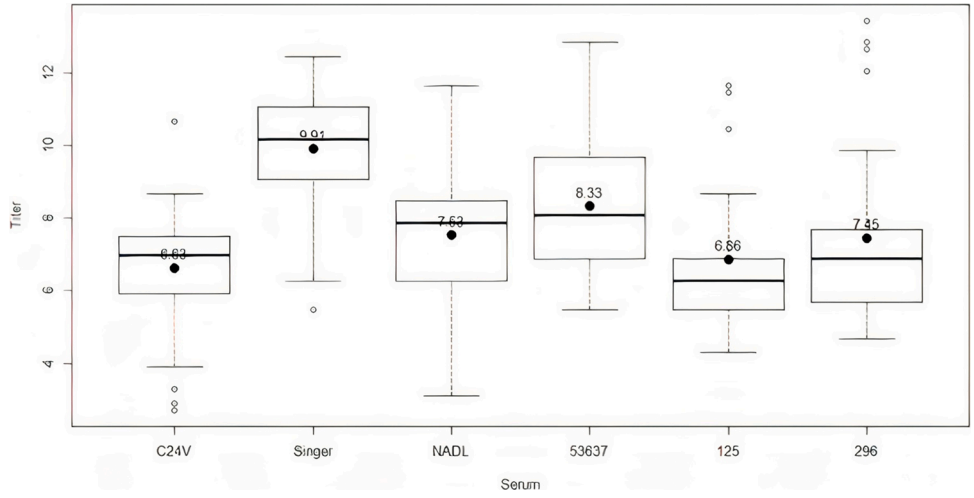

Fig. 2. Box plot for neutralizing antibody titers using 30 BVDV isolates (5 BVDV-1a, 1 BVDV-1d, 6 BVDV-1e, 2 BVDV-1f, 1 BVDV-1g, 3 BVDV-1h, 2 BVDV-1i, 5 BVDV-1k, 4 BVDV-2a, and 1 HoBi) against antisera generated against three BVDV-1a vaccine strains (C24V, Singer, and NADL) and three BVDV-2a strains (53637c, 125c, and 296c).

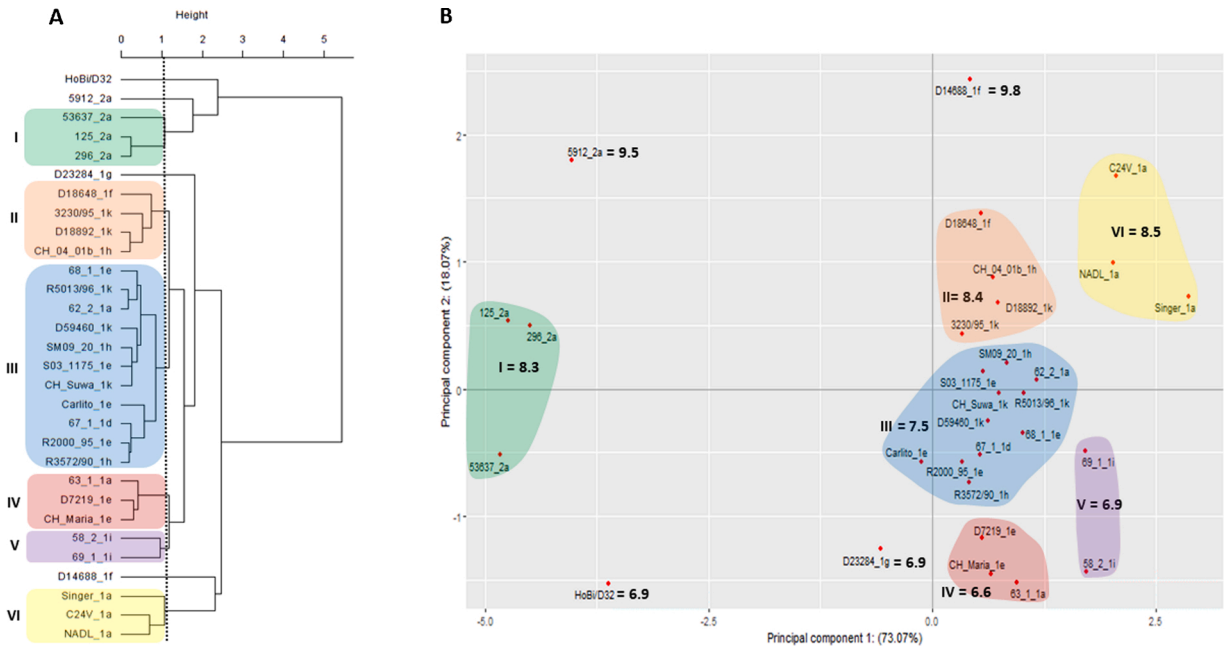

Fig. 3. Methods to evaluate similar antigenic clustering using 30 BVDV strains (5 BVDV-1a, 1 BVDV-1d, 6 BVDV-1e, 2 BVDV-1f, 1 BVDV-1g, 3 BVDV-1h, 2 BVDV-1i, 5 BVDV-1k, 4 BVDV-2a, and 1 HoBi) against antisera generated against three BVDV-1a vaccine strains (C24V, Singer, and NADL) and three BVDV-2a strains (53637c, 125c, and 296c). (A) Cluster analysis dendrogram using Ward's method combing the variation from both principal component 1 and 2 to cluster strains into like groups. (B) Principal component scatter plot displaying independent contribution of the first two principal components accounting for the largest variation in the samples. The mean neutralizing titer of the antigenic cluster is demonstrated along with the cluster identification based on dendrogram A.

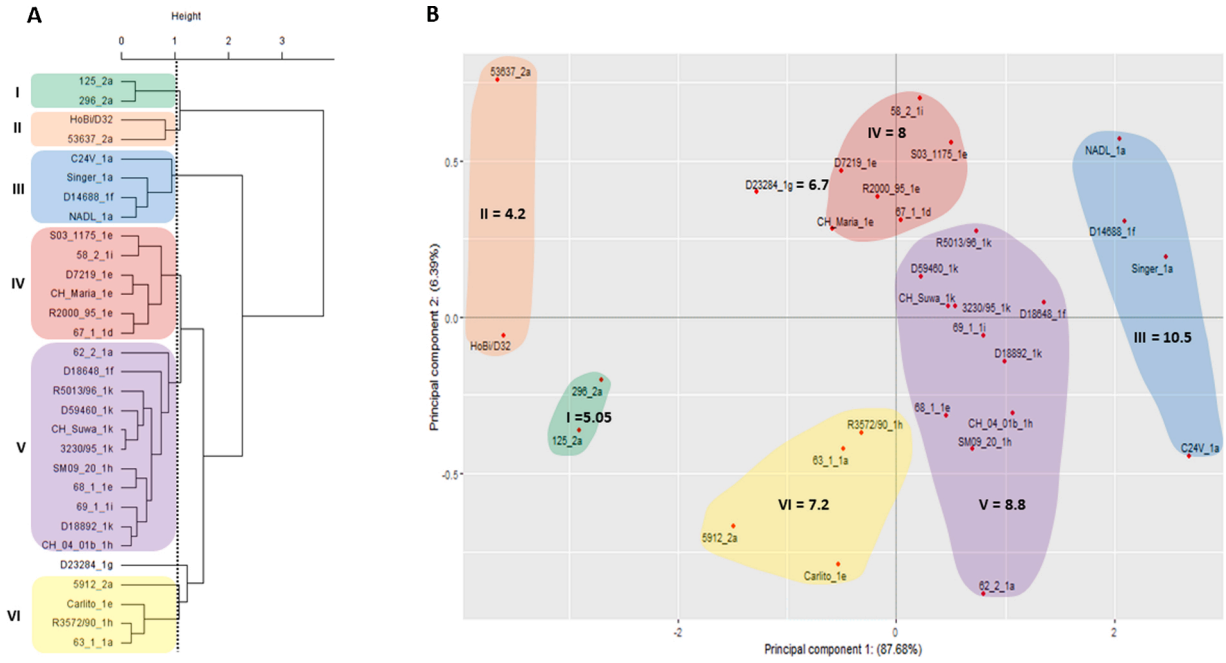

Fig. 4. Methods to evaluate similar antigenic clustering using 30 BVDV strains (5 BVDV-1a, 1 BVDV-1d, 6 BVDV-1e, 2 BVDV-1f, 1 BVDV-1g, 3 BVDV-1h, 2 BVDV-1i, 5 BVDV-1k, 4 BVDV-2a, and 1 HoBi) and antisera generated against three BVDV-1a vaccine strains (C24V, NADL, and Singer). (A) Cluster analysis dendrogram using Ward's method combing the variation from both principal component 1 and 2 to cluster strains into like groups. (B) Principal component scatter plot displaying independent contribution of the first two principal components accounting for the largest variation in the samples. The mean neutralizing titer of the antigenic cluster is demonstrated along with the cluster identification based on dendrogram A.

demonstrated by clusters III, IV, and V that have lower VN titers as they are on the opposing side of the Y axis and are more spatially distant from the cluster containing the BVDV-1 vaccine strains (Fig. 3B).

Interestingly, one vaccine strain (5912_2a) and field isolate (D14688_1f) that lacked proximity to the clusters had high VN titers, which were greater than average VN titers for the vaccine virus clusters for each respective BVDV species (Fig. 3B). Although two of the isolates (HoBi/D32 and D23284_1 g) with distinct spatial proximity did not have the lowest VN titers, the titers in general were lower than the majority of viral clusters (Fig. 3B). The same was observed for cluster V,

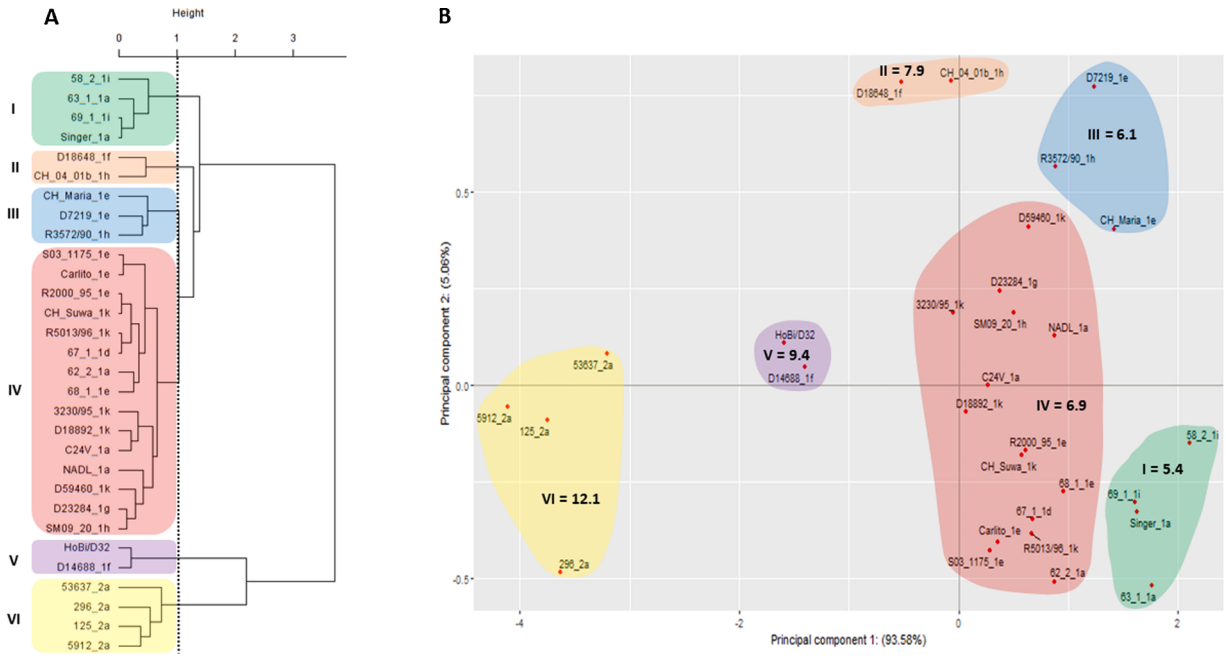

Fig. 5. Methods to evaluate similar antigenic clustering using 30 BVDV strains (5 BVDV-1a, 1 BVDV-1d, 6 BVDV-1e, 2 BVDV-1f, 1 BVDV-1g, 3 BVDV-1h, 2 BVDV-1i, 5 BVDV-1k, 4 BVDV-2a, and 1 HoBi) and three BVDV-2a antisera generated against vaccine strains (296c 53637c, and 125c). (A) Cluster analysis dendrogram using Ward's method combing the variation from both principal component 1 and 2 to cluster strains into like groups. (B) Principal component scatter plot displaying independent contribution of the first two principal components accounting for the largest variation in the samples. The mean neutralizing titer of the antigenic cluster is demonstrated along with the cluster identification based on dendrogram A.

representative of the BVDV-1i isolates, and cluster IV, comprised of BVDV-1e and -1a isolates, that were observed to have some of the lowest titers (Fig. 3B).

To better understand the contribution of each BVDV species-specific antisera to the cross-neutralization patterns associated with the same 30 virus dataset, antigenic comparisons were made using only the BVDV-1 vaccine strains specific-antisera (C24V_1a, NADL_1a and Singer_1a antisera; Fig. 4) as well as only using the BVDV-2 vaccine strains specific-antisera (125_2a, 296_2a and 53637_2a; Fig. 5). These comparisons were evaluated because of the percent variability that was accounted for by PC1 and PC2 for each antisera group (Figs. 4 and 5).

PCA using only the VN data for the BVDV-1 vaccine strains specific-antisera yielded similar trends as observed when using all six vaccine strains antisera, but specific VN titer patterns could be observed. Notable differences observed for the BVDV-1 specific-antisera were larger clusters containing BVDV-1 field isolates and the presence of only one individual branch within the PCA dendrogram. In the dendrogram (Fig. 4A), strain 5912_2a grouped in a cluster with other BVDV-1 isolates (VI). BVDV-2 vaccine strains were separated in two clusters, with cluster I with vaccine strains 125_2a and 296_2a and cluster II with HoBi/D32 clustered along with vaccine strain 53637_2a (Fig. 4A). Cluster 2 was the most spatially divergent group in scatter plot (Fig. 4B).

Viruses that formed individual branches (Fig. 3A) when using all six vaccine strains antisera for comparisons were all contained in clusters with other viruses (Fig. 4A), except for D23284-1g cluster (Fig. 4A), which also formed a single cluster when the six antisera titers were analyzed (Fig. 3A). Vaccine BVDV-1 strains formed one cluster like in the PCA with all the antisera titers (Fig. 3A), but the field isolate D14688-1f, which was previously divergent as a single cluster (Fig. 3A) clustered within the vaccine cluster (III, Fig. 4A). This is suggestive that this isolate is very antigenically similar to the vaccine strains.

It should also be noted that most of the BVDV-1 isolates were observed on the same side of the PC1 axis as the BVDV-1a vaccine strains in the PCA dendrogram, but isolates D7219_1e, R200_95_1e, CH_Maria_1e (IV), Carlito_1e, R3572/90_1 h, 63_1_1a (VI) and D23284_1g were observed on the adjacent side of the PC1 axis in the PCA scatterplot (Fig. 4B). Specifically, these results are noteworthy as they demonstrate the majority of BVDV-1e isolates may be more antigenically different than BVDV-1a vaccine strains as well as the observation that also one BVDV-1a isolate, namely 63_1_1a may also be antigenically divergent albeit is a BVDV-1a isolate and genetically similar to the BVDV-1a vaccine strains.

PCA using only the VN data for the BVDV-2 vaccine strains specific-antisera demonstrated further antigenic differences as specific VN titer patterns could be observed (Fig. 5), similar to observations associated with using only BVDV-1 vaccine strains specific-antisera. Since no non-US BVDV-2 field isolates were included in the study, the dendrogram build with only BVDV-2 antisera titers showed the four BVDV-2a vaccine strains as having a similar behavior in neutralization and belonging to one antigenic cluster (VI, Fig. 5A and 5B). Interestingly, isolates D14688-1f and HoBi/D32 clustered together and average VN titers for these two isolates tended to be higher than other BVDV-1 isolates and strains (Fig. 5), and D14688_1f was not similar to any other isolate when the six antisera were analyzed (Fig. 3A). When using only BVDV-2 antisera results, as for when analyzing only BVDV1 antisera, the highest VN titers among BVDV-1 isolates were observed against isolate D14688_1f. BVDV-1i isolates were grouped into one cluster when all six antisera results were analyzed (Fig. 3A) and a similar trend was observed when using the BVDV-2 specific-antisera (Fig. 5A and 5B). Two BVDV-1i isolates along with two BVDV-1a isolates/strains formed one cluster (I; Fig. 5) and this cluster also had the lowest VN titers as compared to the other BVDV-1 isolates/strains.

4. Discussion

More than a growing number of species, the description of new subgenotypes within BVDV species has been growing expansively in the last years, with at least 22 subgenotypes described for BVDV-1, 3–4 for BVDV-2 and initial subgenotype classification in the HoBiPeV species (Yeşilbağ et al., 2017). In the USA the previously described subgenotypes were 1a, 1b and 2a, but in the last two years 1i, 2b and 2c isolates were firstly detected (Neill et al., 2019a; Neill et al., 2019b).

Commercial vaccines available in the country contain well known 1a and 2a strains in their composition, but cross protection is not well defined within the diverse species.

Finding patterns or relationships between subgenotypes and antigenic properties could help in establishing an antigenic classification for isolates, since genetic classification is easier than antigenic characterization through VN titers. VN assay is the gold standard test for determining BVDV antigenic properties, but interpretation of data can be difficult when multiple isolates are analyzed. The antigenic variability measured by neutralization in VN assays results is mostly due to antibodies against the E2 protein, which is an envelope glycoprotein that is highly variable, immunogenic and induces the production of most virus-neutralizing antibodies (Deregt et al., 1998). However, as observed, E2 gene or amino acid phylogeny is not able to describe antigenicity of isolates, since E2 epitopes that are potential targets for neutralizing antibodies are conformational epitopes subjected to post translational processes, as glycosylation (Li et al., 2013).

Additionally, serological relatedness is typically expressed as coefficient of antigenic similarity (R) using VN titers (Bachofen et al., 2008). The coefficient of antigenic similarity (R) is calculated based on A antiserum titer against B virus strain and B antiserum titer against A virus strain and it generates a scale in which a result closest to 100 means less antigenic differences in between the two viral strains. Thus, as viral titers, this calculation provides a value between the specific viral strain and the corresponding antisera and does not allow for comparisons of antigenic relationships among multiple isolates.

In a previous study (Mosena et al., 2020), a new method for interpretation of VN results used PCA to generate antigenic clustering plots. This method allowed visualization of antigenically similar and divergent BVDV isolates when analyzing multiple isolates against multiple antisera. Thus, the relationship between isolates could be clearly interpreted. In this study, we used the same PCA analysis to antigenically characterize vaccine strains and several field isolates from a variety of subgenotypes and different geographic origins. Some of the Swiss isolates (CH_04_01b_1 h and CH_Maria_1e) used in the current study were previously used for antigenic comparisons against BVDV-1a antisera (Bachofen et al., 2008). R values < 25 are generally considered to indicate significant antigenic differences and R values were previously reported to be > 25 for the CH_04_01b_1 h isolate against BVDV-1a antisera (Bachofen et al., 2008). Therefore, at least one of the previously reported R values for the CH_Maria_1e isolate was > 25 which would suggest these isolates were antigenically similar to the BVDV-1a antisera (Bachofen et al., 2008). Interestingly, in the current PCA, the CH_Maria_1e and CH_04_01b_1 h isolates clustered in separate clusters from each other as well as separate clusters from the BVDV-1a vaccine strains, and this was regardless of antisera used for comparisons and is suggestive of antigenic differences. The Italian isolate D7219_1e was also evaluated previously in a cross neutralization study, where sera generated against vaccines containing only 1a or 1b strain in its composition were tested through VN assay for cross neutralization potential against 1a, 1b and 1e isolates (Sozzi et al., 2020b). No vaccine antisera could generate cross neutralization titers higher than 1/10 for D7219_1e (Sozzi et al., 2020b). In the current PCA, this field isolate did not cluster with 1a and 1b isolates other than 63_1_1a when BVDV-1 antisera were used.

Antigenic relatedness could be observed in the antigenic PCA plots presented in this study. Given that more isolates from several subgenotypes were used for the PCA as compared to the previous study that evaluated only isolates of US origin (Mosena et al., 2020), more marked trends associated with antigenic differences could be more clearly discerned. Since no specific pattern was associated with antigenic relatedness, it is rather dependent on the isolate than the subgenotype, i.e., isolates of the same subgenotype were observed to cluster in various BVDV-1 PCA groups. Dendrogram and scatter plots did highlight that some isolates are antigenically divergent from most isolates described in this study. Isolate D14688_1f and D18648_1f were each positioned in different clusters thus showing different antigenic relationships for each of the PCA scenarios and as well as different antigenic relationship between the two BVDV-1f isolates. This points that some isolates are antigenically very divergent from members of the same species and subgenotype.

However, some subgenotypes presented relationships that remained similar in all the plots regardless of the antisera used for comparisons. Similarly, antigenic patterns could be observed for the BVDV-1i subgenotype. In two out of three scenarios the two 1i isolates (UK isolates 58_2_1i and 69_1_1i) clustered together, sometimes with isolates of other subgenotypes isolates inside the same cluster. This was not observed with other subgenotypes included in the study, where some isolates from the same subgenotype would be clustered separately. Although only two BVDV_1i isolates were included, it should be further analyzed as there may be some feature that this subgenotype might provide to investigate specific antigenic determinants linked to patterns of neutralizing antibodies.

In addition to the illustration that antigenic relationships between isolates could not be linked to subgenotype classification, some of our results highlight previous concerns raised about current vaccines efficacies in protecting against the broad diversity of BVDV. Even BVDV-1a, the most frequent subgenotype worldwide (Yeşilbağ et al., 2017), presented in this study isolates that were antigenically divergent from each other and vaccine strains. It raises questions on the cross protection of 1a vaccine strains against other subgenotypes. Antigenic characterization of pestiviruses has been challenging since the standard VN titer results can be hard to visualize and interpret due to differences in comparison between different antisera and the number of isolates and antisera used. PCA was already shown to be a statistical method that can turn VN titers into an easy and clear interpretation of antigenic relationships between multiple isolates using multiple antisera and can be a tool to identify antigenic patterns. This could become a useful resource to design vaccine with improved efficacy against a broader diversity of BVDV field isolates. In order to turn VN results with PCA interpretation into a tool for the vaccine industry, vaccination and challenge studies are necessary to understand if and how antibody neutralization patterns in vitro are related to cross protection or failure in vivo. Furthermore, VN titers are a measure only for the humoral immune response and provide an understanding of how efficiently the virus is neutralized by antibodies. VN titers do not account for the cell mediated immune response, which is also related to protection observed during in vivo vaccination studies (Ridpath, 2013; Chase, 2013; Platt et al., 2009). Although it is important for immune protection, there is still neither consensus on the role and mechanisms of cell mediated immune responses in BVDV infection, nor does an established method exist for its measurement (Becher et al., 2003; Chase, 2013). Nevertheless, characterization of isolates regardless of their type of immune response will allow for a more directed analysis of the genome and its relationship to immunological and antigenic differences that may exist. We believe the results presented in this study could further lead to a deeper genetic comparison among antigenically similar and dissimilar isolates to determine if there are molecular genetic signatures that could be related to antigenic similarity or divergence between isolates. This might be a tool to improve the composition of current vaccines and broaden its cross protection against diverse pestiviruses, which is important for the success of BVDV control programs.

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Author contributions

Conceived and designed the experiment: ACM, SMF, JDN.
Performed the experiment: ACM, SMF, RPD, JDN.
Analyzed the data: ACM, HM, EC.
Contributed reagents/materials/analysis tools: SMF, RPD, RB, GMD, MS, CWC, JDN.
Wrote the paper: ACM and SMF.
Reviewed the paper: all authors.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2021.114328.

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