Virology 158 (2019) 19–29

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

Identification of BVDV2b and 2c subgenotypes in the United States: Genetic and antigenic characterization

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ARTICLE INFO

Keywords:
Pestivirus
Subgenotype
Cytopathic
Virus neutralizations
Genetic diversity
Antigenic diversity

ABSTRACT

Bovine viral diarrhea virus (BVDV), a ubiquitous pathogen of cattle, causes subclinical to severe acute disease. Two species of BVDV are recognized, BVDV1 and BVDV2 with BVDV1 divided into at least 21 subgenotypes and BVDV2 into 3–4 subgenotypes, most commonly using sequences from the 5' untranslated region (5' UTR). We report genomic sequencing of 8 BVDV2 isolates that did not segregate into the 2a subgenotype; but represented two additional BVDV2 subgenotypes. One BVDV2 subgenotype was previously recognized only in Asia. The other seven viruses fell into a second subgenotype that was first reported in Brazil and the U.S. in 2002. Neutralization assays using antiserum raised against vaccine strain BVDV2a 296c revealed varying degrees of neutralization of genetically diverse BVDV2 isolates. Neutralization titers decreased from 1.8 to more than a four log(2) decrease. This study illustrated the considerable genetic and antigenic diversity in BVDV2 circulating in the U.S.

1. Introduction

Bovine viral diarrhea viruses are members of the *Flaviviridae*, genus *Pestivirus*. Recently, a proposed change of names of the Pestivirus species was presented to provide a more uniform naming system. There are 11 recognized species, A through K, where BVDV1 and BVDV2 are Pestivirus A and B, respectively (Smith et al., 2017). Here, for continuity with current literature, these viruses will be termed BVDV1 and BVDV2. BVDV are ubiquitous viral pathogens of cattle. These viruses are associated with mild, subclinical disease to severe acute hemorrhagic syndrome (Carman et al., 1998; Ridpath et al., 1994; Stoffregen et al., 2000), with the latter a result of infection with a subset of BVDV2 (Ellis et al., 1998). BVDV are also commonly associated with cases of bovine respiratory disease in conjunction with other primary and opportunistic respiratory pathogens (Fulton, 2009; Fulton et al., 2009a; Hay et al., 2016). BVDV infects most organs of the host including respiratory, immune, digestive and reproductive systems. The severe decline in circulating lymphocytes and lymphoid depletion are considered major causes of the immunosuppression associated with BVDV infection (Falkenberg et al., 2014; Kapil et al., 2005; Liebler-Tenorio et al., 2003; Steffen et al., 2014). Additionally, if infection occurs early in pregnancy, the resulting calf may be born persistently infected, spreading the virus throughout its lifetime (Bielefeldt Ohmann et al., 1987; Fulton et al., 2009b; McClurkin et al., 1985).

BVDV is a single-stranded plus-sense RNA virus that encodes a single large open reading frame (ORF). The protein that is translated from this ORF is post-translationally processed by both cellular and viral proteases to yield the mature proteins (Rumenapf et al., 1993; Xu et al., 1997). The virus encodes twelve mature proteins, four structural (capsid, E^{rns}, E1 and E2) and eight nonstructural proteins (N^{pro}, p7, NS2, NS3, NS4a, NS4b, NS5a and NS5b). The E2 envelope glycoprotein is the immunodominant protein found on the surface of the virion against which the majority of neutralizing antibodies are produced (Deregt et al., 1998a; Tijssen et al., 1996). The E2 protein is responsible for binding the cellular receptor and membrane fusion following acidification of the endosome (El Omari et al., 2013; Li et al., 2013; Ronecker et al., 2008; Wang et al., 2014a).

BVDV has been segregated into species (genotypes) and subgenotypes based on viral sequences, most commonly the 5' untranslated region (5' UTR), with some analyses done using N^{pro}, E2 and NS3 sequences (Becher et al., 1999, 1997; Harasawa, 1996; Harasawa and Mizusawa, 1995; Harpin et al., 1995; Nagai et al., 2004; Ridpath et al., 1994; Vilcek et al., 1994). Two species of BVDV are currently recognized, BVDV1 and BVDV2 that are differentiated at both the genetic

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https://doi.org/10.1016/j.virol.2018.12.002
Received 24 September 2018; Received in revised form 3 December 2018; Accepted 3 December 2018
Available online 13 December 2018
0042-6822/ Published by Elsevier Inc.

and antigenic levels (Ridpath and Bolin, 1998; Ridpath et al., 2000). Further, both species are composed of multiple subgenotypes that are differentiated at the genomic level. Sequence analysis of the 5' UTR has resulted 21 (BVDV1a through BVDV1u) subgenotypes as well as four BVDV2 subgenotypes (Yesilbag et al., 2017).

BVDV strains exist as either one of two biotypes, noncytopathic, the most prevalent, and cytopathic. These biotypes are named for their activity in cell culture where the cytopathic viruses kill infected cells and noncytopathic viruses do not. Noncytopathic viruses can establish persistent, non-apparent infections, both in vitro and in vivo. Cytopathic viruses arise from noncytopathic viruses either by incorporation of cellular RNA sequences into the NS2/NS3 coding region or by duplication of the NS3 region (Baroth et al., 2000; Meyers et al., 1991a, 1991b; Muller et al., 2003; Qi et al., 1998, 1992; Rinck et al., 2001; Tautz et al., 1993; Vilcek et al., 2000). Point mutations causing cleavage of the NS2/NS3 are known (Kummerer et al., 1998; Pellerin et al., 1995). The insertion of cellular sequences or duplication of the NS3 region causes elevated levels of the NS3 protein in the infected cell resulting in the cytopathic phenotype (Lackner et al., 2005; Rinck et al., 2001).

Vaccines containing BVDV have been available for more than 50 years. These vaccines provide broad protection against BVDV that is effective from a herd perspective (Ridpath, 2013), but can have limited durations of immunity (Fulton, 2015; Fulton et al., 1995). Limited duration is most often associated with vaccines containing killed or inactivated components. Another short-coming of the current vaccines is their inability to provide sterilizing immunity and reliably prevent fetal infections. It is well established this is particularly true of killed component vaccines (Walz et al., 2018). A meta-analysis examining the efficacy of vaccination to prevent reproductive disease was recently conducted (Newcomer et al., 2015) that compiled results from previously published studies. This analysis revealed that overall, vaccination decreased abortions by 45% and fetal infections by almost 85%. However, a single persistently-infected calf in a herd can have a large impact from the continuous shedding of virus and exposure of herd mates, to the possibility of infecting pregnant cattle (Fulton, 2015). Thus, more efficacious vaccines are warranted.

Previous work has shown that there is antigenic variation amongst BVDV isolates. Initially, antigenic variability of BVDV isolates was investigated using monoclonal antibodies and antisera generated by vaccination (Bolin et al., 1988; Bolin and Ridpath, 1989, 1990). Later, BVDV isolates were identified in Canada that caused severe acute disease (Carman et al., 1998; Corapi et al., 1989). These isolates segregated into a second genotype that was genetically and antigenically distinct from the BVDV1a and 1b (Pellerin et al., 1994; Ridpath et al., 1994), termed BVDV2. BVDV2 infections occurred in herds vaccinated with BVDV1a (Van Campen et al., 2000) and BVDV1a vaccines failed to protect pregnant dams from fetal infection following challenge with a BVDV2 strain (Brock and Cortese, 2001). Antigenic differences between BVDV1a and BVDV2 strains were further illustrated by monoclonal antibody binding assays (Corapi et al., 1988, 1990; Ridpath et al., 1994, 2000). This led to the realization that BVDV2 strains were needed as a component of commercial vaccines to provide broader protection. Since that time, with the improvement and use of DNA sequencing technologies, it became apparent that there were more than the three previously identified subgenotypes of BVDV. In the BVDV1 species alone, there are more than 20 putative subgenotypes (Yesilbag et al., 2017). Four subgenotypes have been suggested for the BVDV2 species (BVDV2a-d) as indicated by comparison of 5' untranslated region (UTR) sequences (Decaro et al., 2016; Flores et al., 2002; Giangaspero et al., 2008, 2001; Jones et al., 2001; Ridpath, 2005). Demonstration of antigenic variation amongst subgenotypes was demonstrated by cross neutralization using antisera raised against specific viruses and monoclonal antibody binding assays (Bachofen et al., 2008; Bolin and Ridpath, 1998; Ridpath et al., 2010).

The amount of genetic and antigenic variation in circulating strains of BVDV make broadly and highly protective vaccines that confer fetal protection a difficult goal. The actual amount of variation is unknown. Because of this, it is also unknown how variation affects the protection afforded by current vaccines against more divergent viruses. Another issue compounding this is the fact that current vaccines do not contain a BVDV1b component, the most commonly isolated subgenotype in the U.S. (Fulton, 2015). There is currently little information regarding the relationships between BVDV vaccine strains and BVDV circulating in cattle herds. There has been no survey that examines the genetic and antigenic diversity of BVDV in circulation, their relationship to vaccine strains and how well these vaccine strains may protect against the more antigenically diverse viruses. The work described here is a first step toward detailing the genetic variability of the BVDV2 and comparing these viruses genetically and antigenically to a vaccine virus that is in current use.

2. Methods and materials

2.1. Isolation of viruses

Bovine viral diarrhea virus (BVDV) strains were isolated from clinical samples submitted to the National Animal Disease Center BVDV laboratory from diagnostic laboratories or practitioners from the mid to late 1980s to 2016. Clinical samples were collected and submitted to the Kansas State Veterinary Diagnostic Laboratory (KSVDL) for virus isolation. Two viruses, 12–149150 and 12–151955–317, were used in this study. BVDV2 were also obtained from the U.S. Meat Animal Research Center (USMARC, Table 1) that were previously reported in a study examining the BVDV isolated from persistently-infected cattle (Workman et al., 2016). BVDV strains were isolated from these clinical samples using standard virus isolation techniques. Briefly, serum samples, buffy coats or tissue homogenates from samples obtained from necropsy were placed on Madin-Darby bovine kidney cells for 1 h at 37 C in a 5% CO_2 atmosphere. The cells were washed to remove the inoculum and the medium was replaced with fresh medium containing 10% fetal bovine serum (BVDV and BVDV antibody-free) and incubation was continued as before for 4–5 days. In the absence of cytopathic effect (CPE), the cells were fixed and stained as previously described (Ridpath et al., 1994). BVDV isolates at KSVDL were routinely genotyped by 5' UTR PCR and sequencing following isolation (Peddireddi et al., 2018).

2.2. DNA sequence analysis

Determination of the subgenotypes of the BVDV isolates was conducted to determine the extent of genetic diversity and numbers of the different subgenotypes present. Initial genotyping of the individual isolates was done using PCR amplification and DNA sequencing of the 5' UTR as previously described (Ridpath et al., 1994). Near full-length genomic sequences of the BVDV2 of interest were obtained using the random primed, sequence independent sequencing protocol as previously described (Neill et al., 2014) using the MiSeq platform (Illumina, Inc., San Diego, CA). The sequences of the individual viruses were assembled using SeqManNGen version 12 (LaserGene, Inc., Madison, WI). Determination of the sequences of the genomic 3' termini were done using 3' RACE. Genomic RNA was polyadenylated with poly(A) polymerase (Invitrogen/ThermoFisher, Inc., Waltham, MA) according to supplier's specifications. First strand cDNA synthesis was done using an anchored poly(T) primer (5'-ACGCTACGTAACGGCATGACAG $TGT_{24}G$) and Superscript III reverse transcriptase (ThermoFisher, Scientific, Inc.). PCR amplification of the 3' end was done using a BVDV2-specific oligonucleotide primer (5'-CTATAGAGAAGTGATAGGGAGAC AGC) and a PCR primer corresponding to first 24 bases of the poly(T) primer (5'-ACGCTACGTAACGGCATGACAGTG). The PCR amplicons were sequenced using the Sanger sequencing platform (Applied Biosystems, Inc., Thermo/Fisher Scientific) using standard chemistries. The

Table 1
Name, Isolation Information and GenBank accession number for viruses used in this study.

Strain	GenBank accession no.	Species/Subgroup	Year of isolation	Country/Region	Strain	GenBank accession no.	Species/Subgroup	Year of isolation	Country/Region
180	HQ174292	BVDV-1a	2010	USA-AL	**5912c**	MH231129	BVDV-2a	1995	USA-MO
296c	MH806436	BVDV-2a	1995	USA-ND	5Y	KT875152	BVDV-2a	2003	USA-SD
446	HQ174292	BVDV-2a	2010	USA-AL	**95–1501**	MH231130	BVDV-2a	1995	USA-WA
793	HQ174302	BVDV-2a	1995	USA-IN	**AU501**	MH231131	BVDV-2a	2006	USA-AL
890	U18059	BVDV-2a	1990	USA-IA	AU526	KF835697	BVDV-1b	2008	USA-AL
1373	AF145967	BVDV-2a	1993	Canada	**AzSpl**	MH231132	BVDV-2a	1997	USA-AZ
1786c	MH231124	BVDV-2c	1989	USA-unk[b]	**B69519c**	MH231133	BVDV-2c	2006	USA-FBS[c]
2139[a]	MH231125	BVDV-2a	1992	USA-IA	**B9497**	MH231134	BVDV-2a	1997	USA-WY
2412	MH231152	BVDV-2c	1989	USA-unk	**BV1907**	MH231135	BVDV-2a	1995	USA-unk
3237	MH231126	BVDV-2b	1990	USA-unk	C413	NC_002032	BVDV-2a	1997	USA-unk
6010	JN380080	BVDV-1a	2010	USA-AL	CN10.2015.821	MG879027	BVDV-2a	2014	Italy
8831	HQ174299	BVDV-2a	2010	USA-AL	D37-13-2_Dup(-)	HG426479	BVDV-2a	2013	Germany
8833	HQ174300	BVDV-2a	2010	USA-AL	D37-13-2_Dup(+)	HG426480	BVDV-2a	2013	Germany
9231	MH806437	BVDV-2a	2004	USA-OK	Egy/Ismailia/2014	KR029825	BVDV-1b	2014	Egypt
10406	MH231123	BVDV-2a	1993	USA-SD	GS5	KJ541471	BVDV-1a	2013	China
14622	MH231151	BVDV-2c	2004	USA-unk	GX4	KJ689448	BVDV-1b	2012	China
24515	AY149216	BVDV-2a	1993	Canada	HB-1511	KX096718	BVDV-2a	2015	China
37621	HQ174303	BVDV-2a	1995	USA-unk	Hercules	JX297517	BVDV-1b	2004	Canada
570152	MH231128	BVDV-2a	1992	USA-IA	HJ-1	KU756226	BVDV-1b	2010	China
08GB44-1	JQ418633	BVDV-1a	unk	Korea	HLJ-10	JF714967	BVDV-2a	2011	China
08Q723	JQ418635	BVDV-2a	unk	South Korea	Hokudai-Lab/09	AB567658	BVDV-2b	2009	Japan
11F011	KC963968	BVDV-2a	2011	South Korea	IAF103	HQ174301	BVDV-2a	1993	Canada
12-149150	MH231148	BVDV-2c	2012	USA-KS	ILLNC	U86600	BVDV-1b	1991	USA-IL
12-151955-317	MH231150	BVDV-2c	2012	USA-KS	JL-1	KF501393	BVDV-1b	2009	China
125c	MH806434	BVDV-2a	1990	USA-IA	**JV14**	MH231136	BVDV-2a	1998	USA-unk
1336H	MH806435	BVDV-2a	2005	USA-KS	JZ05-1	GQ888686	BVDV-2a	2005	China
53637c	MH231127	BVDV-2a	2003	Canada	KE9	EF101530	BVDV-1b	2007	Germany
KZ-91-CP	LC006970	BVDV-2a	1991	Japan	**Short**	MH231149	BVDV-2c	1989	USA
McCart_c	MH806436	BVDV-2a	1989	USA-unk	**Sanderson6319**	MH231144	BVDV-2a	1992	USA-IA
MnFetus	MH231138	BVDV-2a	1991	USA-MN	Singer	DQ088995	BVDV-1a	1974	USA-IA
NADL	M31182	BVDV-1a	1962	USA-IA	USII-S15	KU159365	BVDV-1a	2015	USA-KS
Nebraska	MH231153	BVDV-1b	1990	USA-NE	USMARC-51998	KP941581	BVDV-1b	2014	USA-AL
New York'93	AF502399	BVDV-2a	1993	USA-NY	USMARC-53873	KP941582	BVDV-2a	2014	USA-MO
NRW_12–13_Dup(-)	HG426483	BVDV-2a	2013	Germany	USMARC-53875	KP941584	BVDV-1a	2014	USA-OK
NRW_12–13_Dup(+)	HG426484	BVDV-2a	2013	Germany	USMARC-55476	KP941585	BVDV-2a	2014	USA-OK
NRW_14–13	HG426485	BVDV-2a	2013	Germany	USMARC-55477	KP941586	BVDV-1a	2014	USA-OK
Olwein #12	MH231139	BVDV-2a	1990	USA-IA	USMARC-55922	KP941588	BVDV-1b	2014	USA-unk
Oregon C24V	AF091605	BVDV-1a	1960	USA-OR	USMARC-55924	KP941590	BVDV-1b	2014	USA-OK
Osloss	M96687	BVDV-1b	1967	Germany	USMARC-55925	KP941591	BVDV-1b	2014	USA-MO
p11Q	AY149215	BVDV-2a	unk	Canada	USMARC-55926	KP941592	BVDV-1b	2014	USA-MO
PA	MH231140	BVDV-2a	1992	USA-PA	USMARC-60764	KT832817	BVDV-2a	2014	USA-unk
PA103	KF835700	BVDV-2a	unk	USA-AL	USMARC-60765	KT832818	BVDV-2a	2014	USA-unk
Parker	MH231142	BVDV-2a	1991	USA-unk	USMARC-60766	KT832819	BVDV-2a	2014	USA-MO
PI103	JN380087	BVDV-2a	2010	USA-OK	USMARC-60767	KT832820	BVDV-2a	2014	USA-MO
PI12	MH231147	BVDV-2a	2016	USA-AL	USMARC-60768	KT832821	BVDV-2a	2014	USA-TX
PI28	MH231141	BVDV-2a	2016	USA-AL	USMARC-60779	KT832822	BVDV-2a	2014	USA-unk
PI99	JN380086	BVDV-2a	2010	USA-OK	USMARC-60780	KT832823	BVDV-2a	2014	USA-OK
Potsdam 1600	HG426491	BVDV-2a	2000	Germany	VEDEVAC	AJ585412	BVDV-1b	unk	Hungary
SD1	M96751	BVDV-1a	1992	USA-OH	**Victor301**	MH231145	BVDV-2a	1990	USA-IA
SD1301	KJ000672	BVDV-2b	2012	China	**WiscA**	MH231146	BVDV-2a	1991	USA-WI
SH-28	HQ258810	BVDV-2a	2009	China	XJ-04	FJ527854	BVDV-2a	2004	China
SH2210-17	HG426493	BVDV-2a	2010	Germany	XZ01	MF278651	BVDV-1b	2016	China
SH2210-23	HG426494	BVDV-2a	2010	Germany					

[a] Viruses in bold are those sequenced in this study.
[b] Unk-unknown.
[c] FBS: isolated from fetal bovine serum.

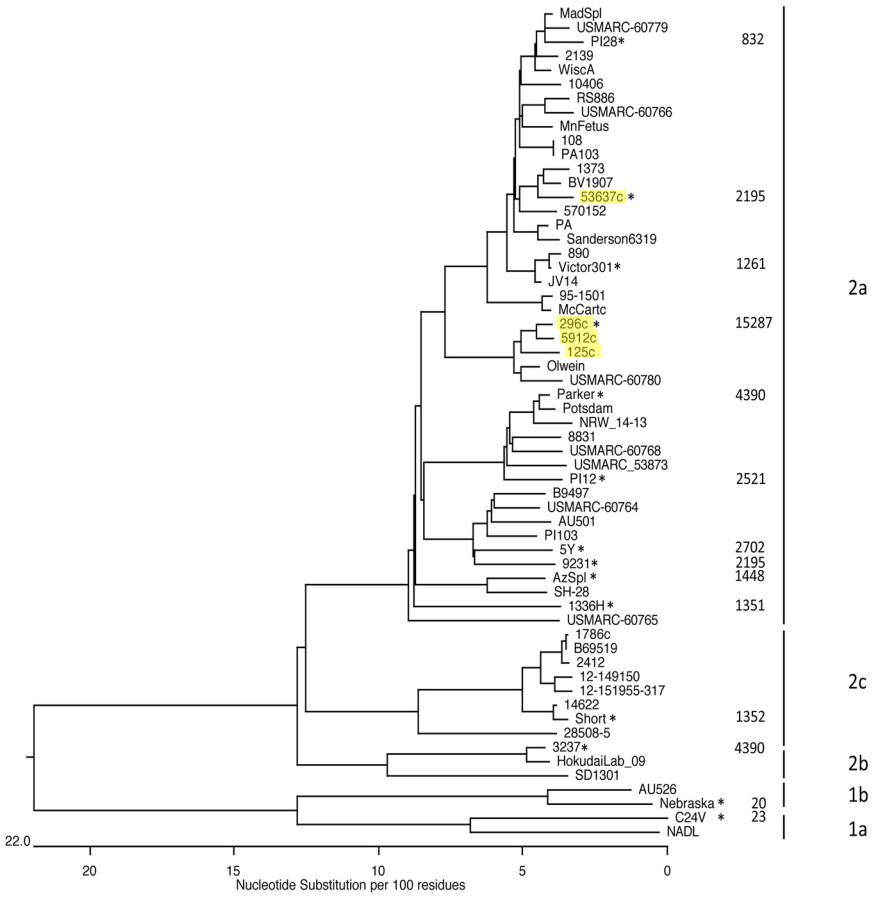

Fig. 1. Phylogenetic analysis of the complete open reading frame (ORF) of BVDV. Phylogenetic analysis of the ORF sequences of 59 BVDV (2 BVDV1a, 2 BVDV1b, 55 BVDV2) strains was conducted to determine genetic relationships. The numbers to the right of certain strains (indicated with a *) are the neutralization titers for these strains using antiserum raised against the BVDV2a vaccine strain 296c. The highlighted strains (yellow) represent strains in current modified live vaccine formulations.

5' UTR sequences were assembled using Aligner (Codon Code, Inc., Madison, WI) and phylogenetic relationships were determined using Molecular Evolutionary Genetics Analysis version 6 (MEGA6) (Tamura et al., 2013).

2.3. Phylogenetic analysis of BVDV2

The viruses used in phylogenetic analyses are shown in Table 1, where the GenBank accession numbers, subgenotype and country of isolation are shown. The year of isolation is given where known, the viruses sequenced in this study have dates of isolation, but the dates of those isolated pre-1990 reflect when they were entered into laboratory inventory. In most of these cases, the specific records were missing. Preliminary phylogenetic analysis was done using the complete ORF sequences of the 35 BVDV2 sequenced here as well as 21 BVDV2 sequences obtained from GenBank using the Clustal W function of MegAlign contained in the LaserGene version 12 package.

A more detailed phylogenetic analysis was done where trees were generated using the complete coding sequences for 103 BVDV strains that were aligned using MUSCLE (Edgar, 2004). For construction of maximum-likelihood trees, MEGA6 was used to determine the most appropriate nucleotide substitution model using the best-fit model tool. The appropriate model was used for phylogenetic inference in PHYML v3 (Guindon et al., 2010) as implemented in Geneious (Kearse et al., 2012). Subtree pruning and regrafting (SPR) was used as the tree searching algorithm and branch support was estimated using 100 bootstrap replicates.

2.4. Antiserum production

Monospecific antiserum was prepared against BVDV2 296c, a cytopathic virus formulated in two modified live vaccines, by intranasal instillation (2.5 ML/nostril, 1×10^6 $TCID_{50}$/ML) into a colostrum-deprived, BVDV and BVDV antibody-free calf. After 21–28 days, the calf received a subcutaneous booster injection of 2 ML of virus at 2 sites. A sample of blood was drawn at approximately 48 days by jugular puncture and serum was prepared and aliquoted and stored at −20C. This serum was used in virus neutralization (VN) assays to determine the homologous 296c neutralization titer. This work was approved by the Institutional Animal Care and Use Committee at the National Animal Disease Center (NADC) under protocol #ARS-2017–673.

2.5. Virus neutralization assays

Virus neutralization (VN) assays were conducted as previously reported (Bolin et al., 1991) using antiserum raised against BVDV2a strain 296c. The antiserum was tested against 296c as well as eight additional, genetically divergent strains of BVDV2. Each antiserum dilution was performed in replicates of five wells. All wells were stained using the E2 protein-specific monoclonal antibody N2 and horseradish peroxidase-conjugated protein G. Final neutralization titers were calculated using the Spearman-Karber method as previously described (Fulton et al., 1995).

3. Results

3.1. Genotyping and genome sequencing of BVDV isolates

A survey to determine the subgenotypes of the BVDV isolates in the inventory of the Bovine Viral Diarrhea Virus Laboratory at the NADC was conducted to determine the extent of genetic diversity and numbers of the different subgenotypes present. A preliminary 5' UTR PCR screening of 524 BVDV isolates yielded a total of 477 usable 5' UTR sequences. These were identified as 123 BVDV1a, 133 BVDV1b and 221

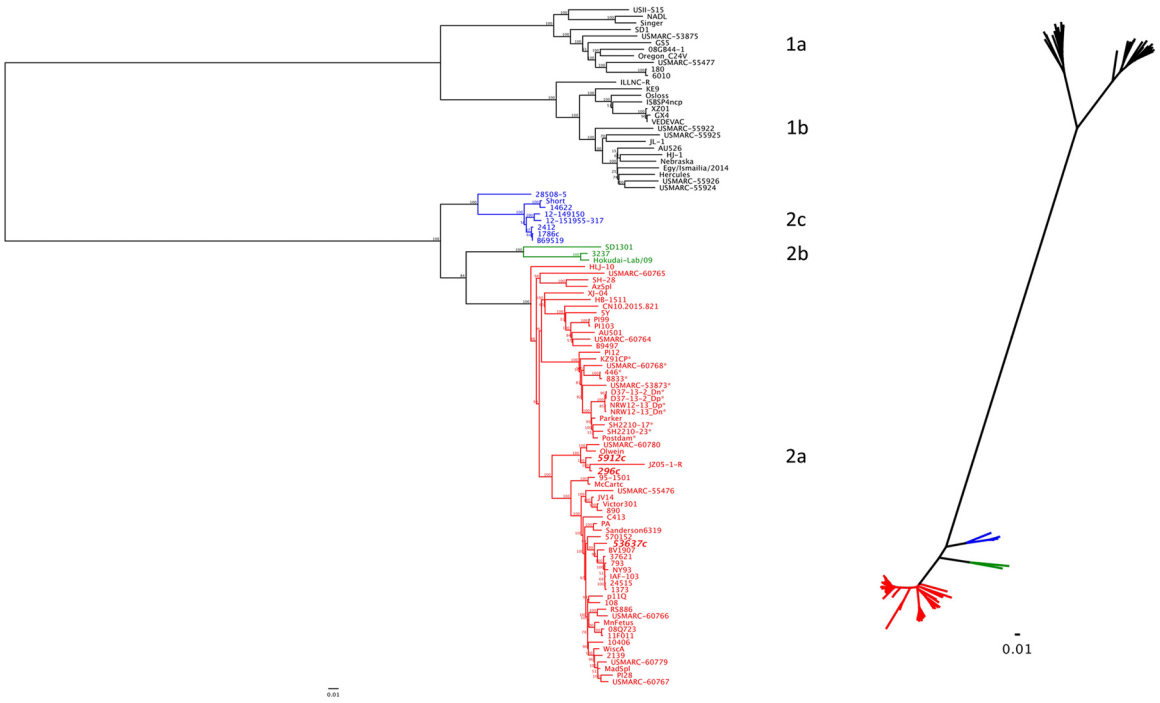

Fig. 2. Maximum likelihood analysis of the BVDV complete ORF. A mid-point rooted ML tree was constructed on the basis of the complete coding RNA sequence of 28 genotype 1 strains and 75 genotype 2 strains. ML phylogeny was carried out using the general time reversible (GTR) model with a gamma shape parameter of 1.94 and a proportion of invariant sites equal to 0.42. The log likelihood of the inferred phylogeny is − 173,933. Numbers indicate the percentage of 100 bootstrap replicates that support each node. The scale bar represents substitutions per site. The colors indicate BVDV2 subtypes and virus names italicized and in bold are current modified live vaccine viruses. Shown to the right is an unrooted tree.

Table 2a
Percent divergence (min-max) based on pairwise distance calculations of the complete open reading frame (ORF).

	BVDV2a	BVDV2b	BVDV2c	BVDV1
BVDV2a	0–14	15[a]–19	15–21	30–34
BVDV2b		1–11	15–18	30–32
BVDV2c			0–11	30–34

[a] Removed JZ05-1 from comparison as it is a known recombinant.

BVDV2. This data, in addition to phylogenetic data from KSVDL, revealed that the majority of these BVDV2 fell within a single, yet genetically variable, BVDV2a subgenotype. However, eight BVDV2 isolates were identified that formed two clades distinct from the main grouping of BVDV2a, forming their own branches (data not shown). Based on these results, genomic sequencing was conducted on 35 BVDV2 isolates with varying amounts of genetic diversity as indicated by their 5' UTR sequences, including the 8 isolates identified above. These isolates are indicated in bold in Table 1. Near full length genomic sequences were obtained for each. As a routine measure, the complete 3' ends of the genomes were determined using 3' RACE. The genomic sequences lacked 0–66 bases at the 5' ends.

A phylogenetic tree was generated from the Clustal W analysis of the 35 BVDV2 complete ORF sequences with 22 BVDV1a, BVDV1b, BVDV2a and BVDV2b ORF sequences previously submitted to GenBank. All sequenced viruses were confirmed to be BVDV2a with the exception of the eight viruses described above. The BVDV2a showed considerable genetic diversity as reflected by the number of nodes and length of branches in this tree. There also appeared to be distinct clades of viruses throughout the 2a subgenotype. The remaining eight viruses were placed into two distinct nodes apart from the 2a viruses (Fig. 1). One virus, 3237, had the highest nucleotide identities (%) with the Japanese isolate HokudaiLab_09 and the Chinese isolate SD1301, both previously reported as BVDV2b based on genome sequencing. The remaining seven were found in a single group distinct from the 2a and 2b clades. This third clade contained BVDV2 28508-5 that was previously reported as BVDV2b based on 5' UTR sequencing (Flores et al., 2002), but is reported here as BVDV2c because of more recent use of 2b for HokudaiLab_09 and the SD1301. This analysis provided further support for significant genetic diversity amongst the BVDV2 (Workman et al., 2016). Interestingly, one BVDV2c isolate, B69519c, was isolated from fetal bovine serum (FBS) during routine testing of FBS lots for purchase.

The ORF sequences of 296c, 5912c, 125c and 53637c, cytopathic viruses that are formulated in commercial cattle vaccines in the U.S. (Fulton, 2015), were included in this tree and are highlighted in Fig. 1. Interestingly, three of these viruses fell into a single group near the middle of the BVDV2a subgenotype that indicated a close genetic relationship amongst these viruses. BVDV2a 53637c was found in a second, adjoining clade. All four of these viruses were located distantly from the more genetically divergent BVDV2a and the two additional subgenotypes.

3.2. Phylogenetic analysis of BVDV2

A more detailed analysis of genetic relationships of 103 BVDV2 ORF sequences with comparison to BVDV1a and 1b viruses was done using MUSCLE and PHYML 3. Using these methods, three monophyletic BVDV2 clades were identified (Fig. 2a) that was similar to that shown in Fig. 1. Each clade was strongly supported by the highest bootstrap values. The ranges of sequence divergence among isolates within clade were ≤ 14% and ≥ 15% between clades (Table 2a). Thirty of the 35 BVDV2 viruses sequenced here fell into a single clade containing prototypic BVDV2a isolates as described above. This clade also contained European and U.S. isolates previously identified as BVDV2c (Jenckel et al., 2014; Workman et al., 2016) and are indicated by asterisks in Fig. 2; however, the sequence divergence between these strains and prototypic BVDV2a strains was ≤ 10%. This showed that these isolates fell into a subgroup within the BVDV2a subgenotype and did not

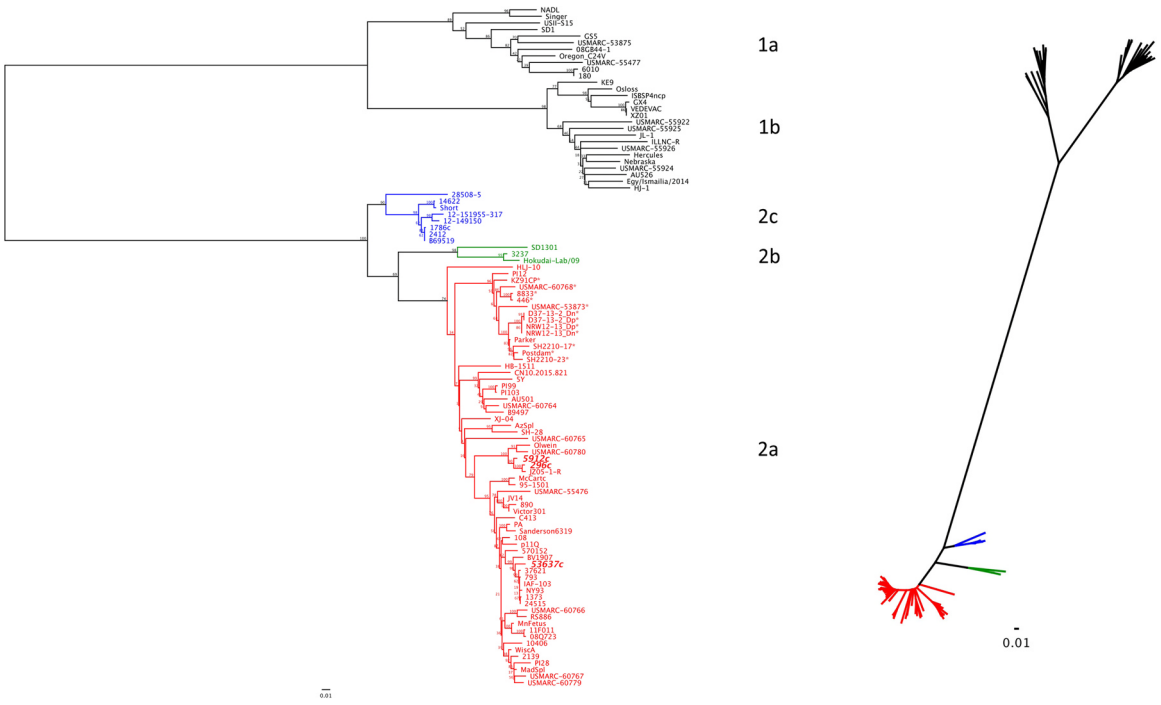

Fig. 3. Maximum likelihood analysis of the BVDV E2 coding sequence. A mid-point rooted ML tree was constructed on the basis of the E2 RNA sequence of 28 genotype 1 strains and 75 genotype 2 strains. ML phylogeny was carried out using the general time reversible (GTR) model with a gamma shape parameter of 1.92 and a proportion of invariant sites equal to 0.31. The log likelihood of the inferred phylogeny is − 19,529. Numbers indicate the percentage of 100 bootstrap replicates that support each node. The scale bar represents substitutions per site. The colors indicate BVDV2 subtypes and virus names italicized and in bold are current modified live vaccine viruses. Shown to the right is an unrooted tree.

Table 2b
Percent divergence (min-max) based on pairwise distance calculations of the E2 coding region.

	BVDV2a	BVDV2b	BVDV2c	BVDV1
BVDV2a	0–13	15–19	15–20	34–40
BVDV2b		2–12	16–19	35–39
BVDV2c			0–12	34–40

represent a unique subgenotype. The second clade contained 3237 from this study and the previously described BVDV2b isolates SD1301 and HokudaiLab_09. Isolate 3237 shared 98.6% sequence identity with HokudaiLab_09. The remaining seven isolates from this study formed the third clade along with BVDV2 28508-5. The isolates in this clade differ from each other ≤ 11% at the nucleotide level; compared to 15–21% between this group and the other subtypes. This is similar to the genetic distance between the previously described BVDV2a and 2b groups (Table 2) as well as distance between BVDV1a and BVDV1b (Fig. 2). The unrooted tree in Fig. 2 also illustrates this point. Thus, based on comparisons of the sequences of the complete ORF from viruses analyzed here, BVDV2 could be divided into three subgenotypes. However, the nomenclature of these subgenotypes was unclear because of conflicting reports where viruses in the two new subgenotypes were reported as BVDV2b (Decaro et al., 2016; Flores et al., 2002) and HokudaiLab_09 identified as a BVDV2b in GenBank. Here, the 3237/HokudaiLab/09/SD1301 subgenotype is referred to as BVDV2b because of the number of isolates using this designation, with the second non-BVDV2a subgenotype referred to as BVDV2c. (Viruses marked with asterisks in Fig. 2a were those that had previously been designated BVDV2c (Jenckel et al., 2014; Workman et al., 2016). From the analysis method used here that included the two additional clades of BVDV2, it appeared that these viruses are in fact BVDV2a, falling cleanly into the BVDV2a portion of the tree.)

Analysis using the 5' UTR, N^{pro} and E2 sequences yielded, for the most part, similar results. This was done to determine whether smaller subgenomic regions could be used to reliably subtype BVDV2 isolates. Different tree topology structures were obtained depending on the genomic region used. The E2 coding region yielded a phylogenetic tree with similar topology to that inferred by the ORF sequences and had the best-supported bootstrap values for the internal nodes of the three subregions for segregation of BVDV2 subtypes (Fig. 3). The percent divergence of BVDV2a in the E2 region ranged from 0% to 13% (Table 2b). The N^{pro} coding region also led to the same subtype determination for isolates with respect to the classification based on the ORF; however, the statistical support for internal nodes separating the subtypes was lower (Fig. 4), with the percent divergence in the BVDV2a ranging from 0% to 14% (Table 2c). The unrooted trees for the E2 and N^{pro} sequences (Figs. 3 and 4) supported this. The tree topology solved for the 5' UTR was significantly different from the trees based on protein coding sequences and low statistical support for internal nodes was observed (Fig. 5). Furthermore, subtype determination based on the 5' UTR provided conflicting results depending on the methods and number of isolates used (data not shown). This was likely due to the short length, lack of nucleotide diversity due to necessity of conservation of specific, functional nucleotides and motifs, and inconsistent gaps introduced during alignments of the 5' UTR compared to other genomic regions analyzed (Becher et al., 1997). The 5' UTR trees (Fig. 5) showed that the two additional clades identified using protein coding sequences were placed instead at the distal end of the 2a viruses, fused to this portion of the tree. This is clearly visible in the unrooted tree. Using these data could lead to the faulty conclusion that these isolates were instead BVDV2a. This observation throws into question the accuracy and utility of the 5' UTR in determining subgroups within BVDV2.

3.3. Cytopathic viruses

The genomic changes that resulted in the cytopathic biotype in seven BVDV2 isolates sequenced in this study were determined. All

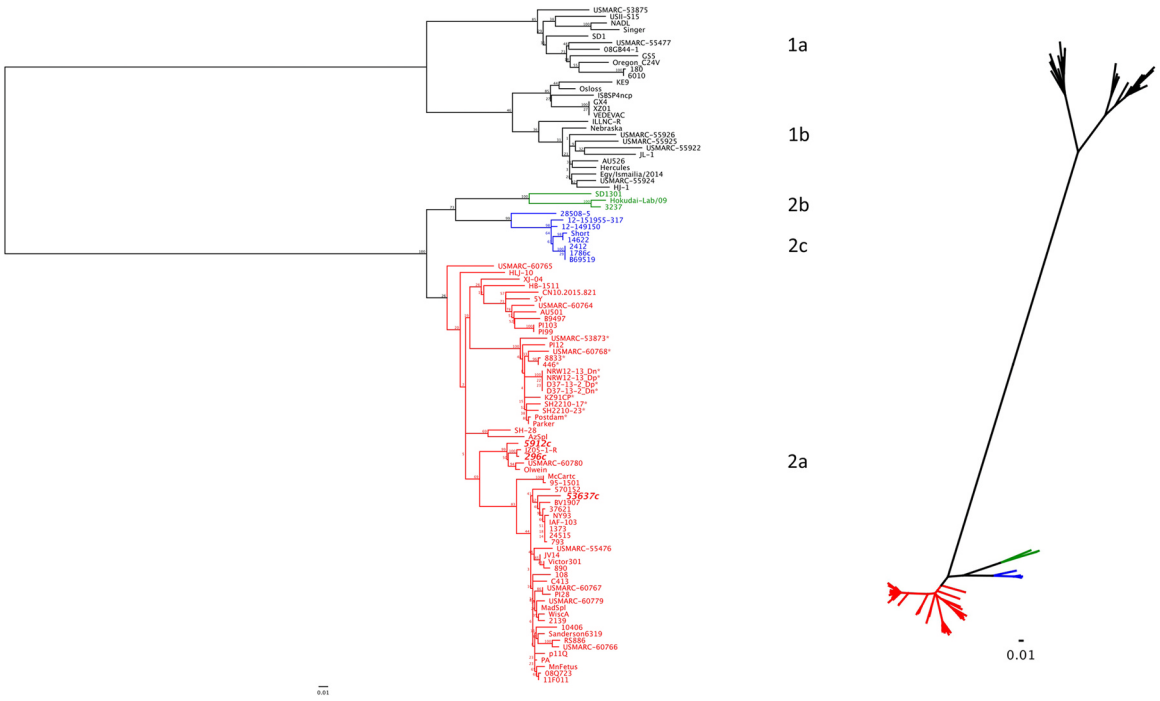

Fig. 4. Maximum likelihood analysis of the BVDV N^{pro} sequence. A mid-point rooted ML tree was constructed on the basis of the N^{Pro} RNA sequence of 28 genotype 1 strains and 75 genotype 2 strains. ML phylogeny was carried out using the Tamura-Nei, 93 (TN93) model with a gamma shape parameter of 1.75 and a proportion of invariant sites equal to 0.37. The log likelihood of the inferred phylogeny is − 7351. Numbers indicate the percentage of 100 bootstrap replicates that support each node. The scale bar represents substitutions per site. The colors indicate BVDV2 subtypes and virus names italicized and in bold are current modified live vaccine viruses. Shown to the right is an unrooted tree.

Table 2c
Percent divergence (min-max) based on pairwise distance calculations of the N^{pro} coding region.

	BVDV2a	BVDV2b	BVDV2c	BVDV1
BVDV2a	0–14	15–18	13*–19	28–35
BVDV2b		2–11	16–18	31–35
BVDV2c			0–9	29–34

were found to contain the jiv insert (Rinck et al., 2001) in the NS2/NS3 coding sequences. The size and borders of the inserted sequences varied amongst isolates, ranging from 272 bases in 125c to 400 bases in 53637c (Fig. 6). The insert within 125c was previously shown to have been the result of recombination with BVDV1a NADL (Ridpath and Bolin, 1995). Few nucleotide differences were noted amongst all six viruses in the jiv insert. This indicated that these viruses were most likely isolated shortly after recombination with the cellular transcript before many nucleotide changes could be introduced by multiple animal infections. The incorporation of the jiv insert would have resulted in conversion to the cytopathic biotype and would most likely have resulted in mucosal disease in the persistently infected animal, indicating their presence.

3.4. Virus neutralizations

VN assays were conducted using bovine antiserum produced following infection with BVDV2a 296c. This analysis was done using 296c along with 11 genetically diverse BVDV2a isolates to determine the extent of cross-reactivity. The homologous neutralization titer of this antisera was 1:15,287 (Table 3). Neutralization of unrelated, antigenically diverse BVDV2 showed a range of lower neutralization titers. Five viruses, 1336H, Short, PI28, Victor301 and AzSpl, had ≥ 3.0 log (2) decreases in titers (8-fold). The lowest neutralization titer, 1:832, was that obtained for PI28, representing a decrease of greater than 18-fold. The average decrease in neutralization titers for all viruses tested was 2.9 log(2) (7.5-fold). Surprisingly, there was a higher neutralization titer with 3227 (1:4390) than observed with the other viruses tested. This titer was identical to that obtained with Parker, a BVDV2a that was more similar to 296c. This possibly indicated the existence of similarity in neutralization epitopes. The neutralization titers for these viruses were also included in Fig. 1 next to the appropriate virus in the tree. The neutralization titers dropped with increasing genetic distance from 296c. Viruses that were found within the same BVDV2a clade (5Y and 1336H, Parker and PI12) had different neutralization titers but titers that were within 2-fold. Neutralization titers of C24V (BVDV1a) and Nebraska (BVDV1b) were very low at 1:23 and 1:20, respectively. These results indicated that considerable differences in antigenicity existed between these genetically variable BVDV2.

4. Discussion

The existence of BVDV2 in the United States was established in the early 1990's with the isolation of severe acute BVDV2 first isolated in Canada (Carman et al., 1998; Ellis et al., 1998) and subsequent genetic and antigenic characterization (Deregt et al., 1998b; Pellerin et al., 1994; Ridpath et al., 1994). Since that time, many strains of BVDV2 have been isolated world-wide. The existence of differences in antigenicity amongst BVDV2 strains were demonstrated from studies using monoclonal antibodies (Ridpath et al., 1994, 2000), but a systematic study of the extent and depth of the existing antigenic differences is lacking. This information is important for cattle producers, veterinarians and biologics companies that use and produce vaccines to protect against BVDV infection. For the fulfilment of the goal of protective vaccination, information concerning antigenic diversity is necessary for both the selection of broadly protective vaccine strains and formulation of effective vaccination strategies. To this end, a preliminary comparison of the complete ORF sequences of 59 viruses was done (Fig. 1). Segregation of viruses into three subgenotypes was evident with both

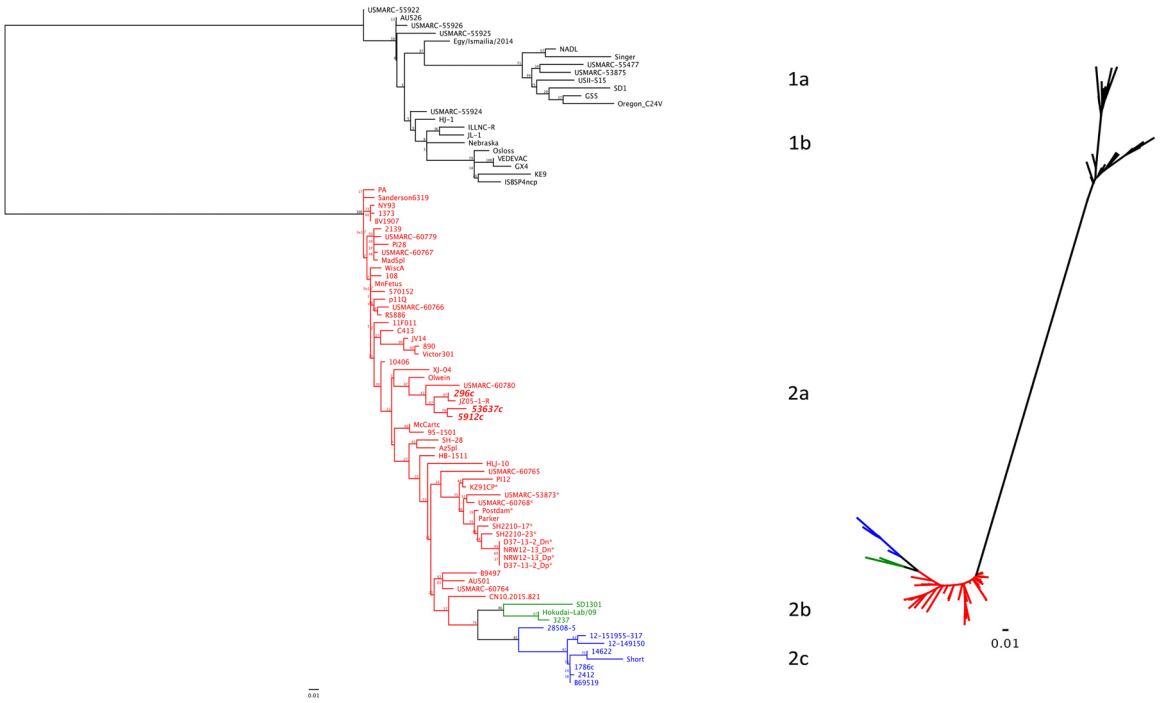

Fig. 5. Maximum likelihood analysis of the BVDV 5' UTR. A mid-point rooted ML tree was constructed on the basis of the 5' UTR of 23 genotype 1 strains and 64 genotype 2 strains. ML phylogeny was carried out using Kimura's 2 parameter model (K2) with a gamma shape parameter of 0.25. The log likelihood of the inferred phylogeny is − 3874. Numbers indicate the percentage of 100 bootstrap replicates that support each node. The scale bar represents substitutions per site. The colors indicate BVDV2 subtypes and virus names italicized and in bold are current modified live vaccine viruses. Shown to the right is an unrooted tree.

the 2b and 2c subgenotypes clearly distinct from the BVDV2a. The BVDV2a were the most prevalent. The seven BVDV2c viruses are the only examples of this subgenotype reported to date. Additionally, BVDV2b 3237 is the only example of this subgenotype in the U.S. This analysis clearly demonstrated the high degree of genetic variation present in BVDV2 viruses in circulation, as illustrated by the number of nodes and length of the branches that were present in this tree. However, it is recognized that this represents a preliminary comparison.

Similar results were obtained in trees generated using a more detailed method of analysis and a larger number of sequences. Trees generated using the ORF, E2 and N^{pro} coding sequences (Figs. 2–4, respectively) provided a detailed overview. Support for the observation that BVDV2 could be placed into three distinct subgenotypes was provided by trees generated using all three protein coding regions. The tree generated using ORF sequences was the most strongly supported tree statistically with very high bootstrap values at almost all nodes. The E2 and N^{pro} trees were very similar to the ORF tree, showing the three subgenotypes but at reduced bootstrap values. All three of the coding regions segregated BVDV2 into three subgenotypes. Conversely, the tree generated using 5' UTR sequences indicated that this region may be unsuitable for subgenotyping BVDV2. The statistical scores of the tree nodes were, for the most part, very low. The two additional subgenotypes, identified using protein coding sequences, were located on the distal end of the BVDV2a trees (Fig. 5). Also, using the 5' UTR sequences, the BVDV1a were presented in the middle of the BVDV1b portion of the tree. Similar results were obtained by Tajima (2004) in phylogenetic analysis of BVDV1 subgenotypes where BVDV1c viruses were identified as a unique subgenotype using the E2 coding sequences while the same 1c viruses were included amongst the BVDV1a using the 5' UTR sequences. The conclusion was that the E2 protein was more useful for classification of subgenotypes within BVDV1. The configuration of these trees in Fig. 5 would lead to the incorrect conclusions that the two BVDV2 subgenotypes were genetically distant BVDV2a and that there was a single tree for the BVDV1. The 5' UTR sequences are reliable for the segregation of BVDV into species but are generally questionable for the further classification of viruses into subgroups, especially in determining subgenotypic relationships as demonstrated here and by others (Becher et al., 1997; Tajima, 2004; Workman et al., 2016).

In light of these results, the question is raised as to which region would be best suited for intra-species classification that provides reproducible and accurate results and is feasible for most BVDV laboratories. Ideally, the complete genome would be used for phylogenetic assignments but this, in many cases, is impractical and difficult to achieve. One observation from generation of bootstrap trees was the declining statistical support as the length of the region used grew shorter. A similar observation was reported from a study of the phylogenetic parameters of hepatitis E virus (Purdy and Sue, 2017) examining whether subgenomic sequences could provide accurate phylogenetic results and, if so, which regions to use. One result was that as the sequences in the comparison became shorter, the greater the loss of phylogenetic information due to the greater likelihood of identical sequences amongst viruses. Additionally, as the sequence shortened, the bootstrap support decreased and trees from short sequences were more likely to differ from trees generated with longer sequences. In the case of BVDV, the use of the short 5' UTR sequences was compounded by the presence of functionally conserved nucleotides. These findings must be taken into consideration when determining the sequences used in BVDV phylogeny.

The N^{pro} region may find the most utility in genotyping these viruses. N^{pro} is short but appears, at least at this point, reliable for future subgroup comparisons. This region had the lowest statistical scores of node junctions but provided accurate classification results when compared to ORF and E2 trees. The N^{pro} can be amplified and sequenced with little trouble, with the N^{pro} being roughly 50% larger than the commonly used 5' UTR. The use of the N^{pro} region is accurate segregating BVDV2 into subgenotypes and provides the same results as the E2 coding sequences (Becher et al., 1999).

The generation of the bootstrap trees using the three protein coding regions also revealed that viruses previously categorized as BVDV2c

Fig. 6. Alignment of jiv sequences present in the NS2/NS3 region of cytopathic viruses sequenced in this study. Alignment of jiv sequences from six cytopathic BVDV2 showing nucleotides in common with all inserts as well as the variable ends. The longest insert was from 53637c and the shortest insert was found in 125c. The numbers at the bottom right show length of inserts.

Table 3
Virus neutralization titers for genetically diverse BVDV2 strains as determined using antiserum raised against BVDV2a 296c.

BVDV2	Neutralization Titer[a]	Log(2) Titer	Log(2) Titer Decrease[b]
296c	15,287[c]	13.9	0
53637c	2195	11.1	2.8
1336H	1351	10.4	3.5
5Y	2702	11.4	2.5
PI12	2521	11.3	2.6
Parker	4390	12.1	1.8
AzSpl	1448	10.9	3.0
9231	2195	11.1	2.8
Short	1352	10.4	3.5
3237	4390	12.1	1.8
PI28	832	9.7	4.2
Victor301	1261	10.3	3.6
Nebraska (1b)	20	4.5	9.4
C24V (1a)	23	4.3	9.6

[a] Reciprocal of the antiserum dilution neutralizing test virus.
[b] Decrease in neutralization titer from the homologous 296c titer.
[c] 296c homologous antiserum neutralization titer.

(Decaro et al., 2017; Jenckel et al., 2014; Workman et al., 2016) were in reality BVDV2a. In trees utilizing all three coding regions, these viruses fell clearly into the BVDV2a subgenotype (Figs. 2 through 4, marked with asterisks) forming a distinct clade within the BVDV2a. This illustrates a weakness in many phylogenetic analyses. Novel sequences are often compared to too few sequences of known strains with limited genetic diversity, resulting in placement in a new subgenotype, when, in reality, viruses should be classified within an existing subgenotype. Standards are needed to determine number of sequences used in the analyses, and the extent of diversity amongst the known sequences. Additionally, many times, only partial regions are sequenced and used, which depending on the region used, may give inaccurate results if the sequences are highly conserved amongst BVDV. Standards are also needed to provide guidance as to which genomic region(s) should be used, length of sequence required and percent pair-wise identity cutoffs for establishment of genotypic divisions and nomenclature guidelines. As an example, both non-2a subgenotypes shown here contain viruses that were designated BVDV2b in earlier publications (Flores et al., 2002; Wang et al., 2014b). Because of advancements in sequencing technologies, it is easier to sequence viruses, either partially or totally. The numbers of BVDV sequences will continue to increase rapidly and correct phylogenetic assignments require these standards.

Included in the phylogenetic trees presented here were four cytopathic BVDV2a strains found in commercial vaccines (highlighted in Fig. 1, italicized and bold font in Figs. 2–5) to determine how they compared genetically to other BVDV2. Three strains, 125c, 296c and 5912c, were found clustered in a single node near the middle of the tree with the fourth virus, 53637c located in an adjacent clade. The cluster of three genetically similar vaccine strains illustrates that there is little genetic diversity in current bovine vaccines containing BVDV2 with each providing essentially equivalent antigenic protection against the

more genetically diverse viruses in circulation. Vaccine strain 53637c showed a 2.8 log(2) decrease (7-fold) in neutralization titer from 296c indicating a difference in antigenicity. Further antigenic comparisons using 296c antiserum showed considerable variation as all heterologous viruses tested had decreased neutralization titers. The titer differences are not as large as that seen when comparing BVDV1 to BVDV2, however; these results suggested that the vaccine strains in current use may not provide sufficient immunity to protect against distantly related BVDV2 strains, particularly as it relates to fetal infections.

The work presented here represents a preliminary study examining genetic and antigenic differences in field isolates of BVDV2. The differences between these strains demonstrated the need for a more in-depth examination of field strains. This includes sequencing of more isolates and a more comprehensive series of titrations using antisera produced against select viruses to determine the effect of sequence variance on antigenicity. The current data suggests one possible outcome would be that a single virus from each subgenotype may be incapable of producing a broadly protective immune response. A single virus may not contain sufficient antigenic information to provide broad protection within a BVDV species. However, it is possible that some strains of BVDV are capable of conferring greater cross-protection. Elements that provide cross-protection need to be identified for a greater understanding of this. Previous data has suggested that single BVDV isolates can confer cross-protection (Grooms et al., 2007; Leyh et al., 2011; Xue et al., 2009). However, the relationship between cross-neutralization and cross-protection is unknown. Further studies are necessary to provide insight into potential deficits in protection derived from vaccines containing single BVDV1a and BVDV2a strains.

Acknowledgements

The authors would like to thank Kathy McMullen, Renae Lesan and Sharon Stark for their excellent technical support. Thanks are also given to Dr. David Alt of the NADC Genomics Laboratory for DNA sequencing assistance.

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