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Genomic and antigenic characterization of a cytopathic bovine viral diarrhea virus 1i isolated in the United States☆

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ABSTRACT

Bovine viral diarrhea viruses (BVDV) are a common global viral pathogen of ruminants. Considerable genetic variability is found amongst BVDV1 isolates, with at least 21 subgenotypes being described. In the United States, BVDV1a and 1b are the only subgenotypes described to date. Here, the genomic sequence of CA2005, a cytopathic BVDV1, was determined. This virus, isolated in California, did not segregate into either BVDV1a or 1b subgenotypes. BLAST analysis showed CA2005 was most closely related to BVDV1i isolates. CA2005 was also the first cytopathic BVDV1i and one of few non-1a, non-1b cytopathic viruses reported. The genomic sequence was 15,752 nucleotides in length. Cytopathogenicity was conferred by duplication of the NS3 protein with a small ubiquitin B insertion at the border of the NS2/NS3 proteins. Virus neutralization assays using antisera against BVDV1a vaccine viruses revealed variable neutralization, suggesting modified live vaccines may not be totally protective against CA2005 and similar viruses.

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. These viruses are associated with varying severity of disease, ranging from subclinical to severe acute (Carman et al., 1998; Ridpath et al., 1994). The viruses are composed of a single-stranded, plus-sense RNA genome that is neither capped at the 5′ end nor 3′ polyadenylated. This genomic RNA encodes a single, large open reading frame (ORF) that is translated to produce a large polyprotein containing the 12 viral proteins in the order N^{pro}-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4a-NS4b-NS5a-NS5b. Post-translational processing by both cellular and viral proteases produces the mature proteins (Rumenapf et al., 1993; Stark et al., 1993; Xu et al., 1997). The ORF is flanked by non-coding regions, the 5′ UTR that contains the internal ribosome entry sequences (IRES) that directs the ribosome in translation of the ORF and the 3' UTR that is involved in viral replication (Chon et al., 1998; Deng and Brock, 1993; Frolov et al., 1998; Pestova and Hellen, 1999; Yu et al., 1999).

BVDV strains exist as either one of two biotypes, the cytopathic or noncytopathic. As their names suggest, cytopathic viruses kill infected epithelial cells in culture where the noncytopathic viruses establish nonapparent chronic infections. Cytopathic viruses arise from noncytopathic viruses by incorporation of portions of cellular transcripts, generally at or near the border of the NS2 and NS3 proteins, that results in the cleavage of NS3 from NS2 or by genomic duplications that result in expression of the NS3 protein singly (Qi et al., 1998; Rinck et al., 2001; Tautz et al., 1993, 1996). Cytopathic BVDV1 generally have a portion of a cellular ubiquitin transcript incorporated, but not exclusively, while BVDV2 cytopathic viruses possess a portion of a cellular DnaJ protein, termed jiv (Rinck et al., 2001). Additionally, cytopathic viruses that contain cryptic point mutations that result in the cleavage of the NS3 from the NS2 are known (Kummerer and Meyers, 2000).

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Phylogenetic studies of BVDV have used multiple regions of the BVDV genomic RNA to compare genetic differences between isolates. The 5′ UTR has primarily been used for this purpose because of its sequence conservation but analyses have also been conducted using the Npro, E2 and NS3 regions that also segregate BVDV into subgenotypes (Becher et al., 1999; Harasawa, 1996; Neill et al., 2019; Ridpath et al., 1994; Vilcek et al., 1994). Using the 5' UTR, multiple subgenotypes of BVDV in both BVDV1 and BVDV2 have been identified. To date, 21 subgenotypes within the BVDV1 (1a-1u) and 3 subgenotypes of BVDV2 have been proposed (Neill et al., 2019; Yesilbag et al., 2017). In the United States, only two BVDV1 subgenotypes have been reported, BVDV1a and 1b. BVDV that fall outside these currently recognized subgenotypes are of interest because it is not known how well current vaccines protect against these more divergent viruses.

A seven year-old Limousine cow from a closed herd that was unvaccinated since 1988 was found dead in its pasture and was submitted to the Veterinary Diagnostic Laboratory at the California Animal Health and Food Safety System at Davis, CA in June of 2005 for determination of cause of death. BVDV was detected in all tissues examined using both fluorescent antibody and immunoperoxidase staining methods. Also observed with the BVDV infection was multifocal or ulcerative esophagitis and enterocolitis with multifocal crypt epithelial necrosis, suggestive of mucosal disease. A BVDV was isolated using standard procedures (McClurkin et al., 1985) and was found to be of the cytopathic biotype. This isolate, CA2005, was determined to be a BVDV1 that did not segregate into either the 1a or 1b subgenotypes by PCR (Ridpath et al., 1994) and sequencing of the 5′ UTR (data not shown). Full genome sequencing was done using a random-primed, sequence independent method as previously described (Neill et al., 2014) using the Ion Torrent sequencing platform. Assembly with a non-cytopathic BVDV1i genome as template resulted in a near full-length genomic sequence of the virus that corresponded to the noncytopathic form of the virus. Using a previously described PCR procedure (Qi et al., 1992), two PCR reactions were performed that allowed determination of the genomic changes that resulted in the cytopathic phenotype. The first PCR reaction, utilizing primers 10 and 11, amplified the NS2/NS3 junction to determine if a cellular mRNA insert at this position conferred cytopathogenicity. The second reaction utilized primers that amplified only if there was a duplication of the NS3 region. This second reaction, utilizing primers 11 and 14, does not result in an amplification product without NS3 duplication. All three primers were modified to match the CA2005 sequences. The sequences of the primers were 10: 5′-AGACTTCATGTACTACATGC; 11: 5′-TTGCCAGAGCAATAACAGGTT AAC; 14: 5′-TCCCAATGGTCACGGACATATACACC. The first reaction using primers 10 and 11 resulted in a small product of approximately 300 bp that did not contain a cellular insert. The second, utilizing primers 11 and 14, resulted in a PCR product of approximately 600 bp. This amplicon was sequenced and revealed duplication of the NS3 region as well as the insertion of a portion of a ubiquitin B (UbiB) mRNA. Inclusion of the duplicated sequences of the genome allowed assembly of the cytopathic virus genome. When using this cytopathic virus sequence in template-assisted assembly using the Ion Torrent sequencing data, full coverage of this cytopathic assembly containing the NS3 duplication was observed. To confirm the full sequence of the 3′ UTR, 3′ RACE was conducted as previously described (Neill et al., 2019) using a gene-specific primer derived from the 3′ end of the RNA-dependent RNA polymerase coding sequences (5′-AGCCGGAAGTCACTCCATCAA CAC). The CA2005 genomic RNA was submitted to GenBank and has the accession number MK775204. This genome was 15,752 nucleotides in length (approximately 62 nucleotides were not determined at the 5′ end of the genome) and possessed a duplication of the NS3 coding sequences with the UbiB mRNA insert of 463 bases located between bases 8330 and 8792. When genomic sequences were compared to other BVDV genomic sequences in GenBank, the closest relative was determined to be ACM/BR/2016, a BVDV1i isolated in Brazil (Mosena et al., 2017). These viruses had a nucleotide identity of 94.4% (not including the UbiB insert in CA2005). The relationship of CA2005 with BVDV isolates and with other pestiviruses was determined. The identity and GenBank accession numbers of all viruses used in this analysis are shown in Table 1. Fig. 1 illustrates the neighbor-joining phylogenetic tree using the Clustal W alignment of the N^{pro} coding sequences of these viruses. This analysis demonstrated that CA2005 (in the shaded blue box) was more closely related to ACM/BR/2016 and that these viruses clustered distinctly from the other BVDV1 subgenotypes. A similar phylogenetic analysis examining possible antigenic relationships using E2 amino acid sequences was not done. This was due to the lack of E2 sequences for non-1a, non-1b viruses as most previous analyses were done using 5′ UTR sequencing. There were not enough sequences available to draw meaningful conclusions.

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

Pestivirus strain	Genotype	GenBank accession no.
USII–S15	1a	KU159365
C24V	1a	AF091605
SD-1	1a	M96751
Singer	1a	DQ088995
AU526	1b	KF835697
Nebraska	1b	MH231153
Osloss	1b	M96687
Hercules	1b	JX297517
10JJ-SKR	1d	KC757383
BJ-2016	1d	MH490943
R2000-95	1e	AY895002
SLO-2407-2006	1e	KX577637
Carlito	1e	KP313732
Maria	1e	AY895003
LN-1	1f	KT896495
SLO-1170-2000	1f	KX987157
CH-04-B2	1 h	EU180039
UM/126/07	1 h	LT631725
ACM/BR/2016	1i	KX857724
CA2005	1i	MK775204
Suwa-ncp	1 k	AY894998
28508–5	2c	AF145968
Short	2c	MH231149
Hokudai-Lab_09	2b	AB567658
SD1301	2b	KJ000672
McCart	2a	MH806436
PA	2a	MH231140
296c	2a	MH806436
D32_00_HoBi	HoBi-like	AB871953
Th_04_KhonKaen	HoBi-like	NC_012812
Coos_Bay	BDV	KJ463422
Pronghorn	Pronhorn	NC_024018

This is the first report of a United States BVDV1 isolate that did not segregate within either the BVDV1a and 1b subgenotypes. Little information was available regarding how cross-reactive an immune response against BVDV1a strains in commercial vaccines are against more divergent strains. The level of cross-reactivity of antisera raised against BVDV1 vaccine strains are possible indicators of vaccine efficacy. To examine antigenic cross-reactivity, bovine antisera were raised against BVDV1a strains C24V, Singer and NADL, all viruses currently formulated in commercial vaccines, and used in virus neutralization assays as previously described (Neill et al., 2019). Neutralization assays were done to determine homologous neutralization titers as well as neutralization titers for CA2005. Table 2 shows the results of these assays. Two of the antiserums, C24V and NADL had rather poor cross-neutralizing titers against CA2005 with decreases of 12 and 24-fold from the homologous titers, respectively. These sera also had the lowest homologous neutralizing titers. The antiserum raised against Singer showed the greatest level of cross-neutralization with 4.5-fold decrease from its homologous titer but still had a cross-neutralization titer of greater than 1:1200 against CA2005. However, it remains to be determined how protective these antibody titers would be against CA2005

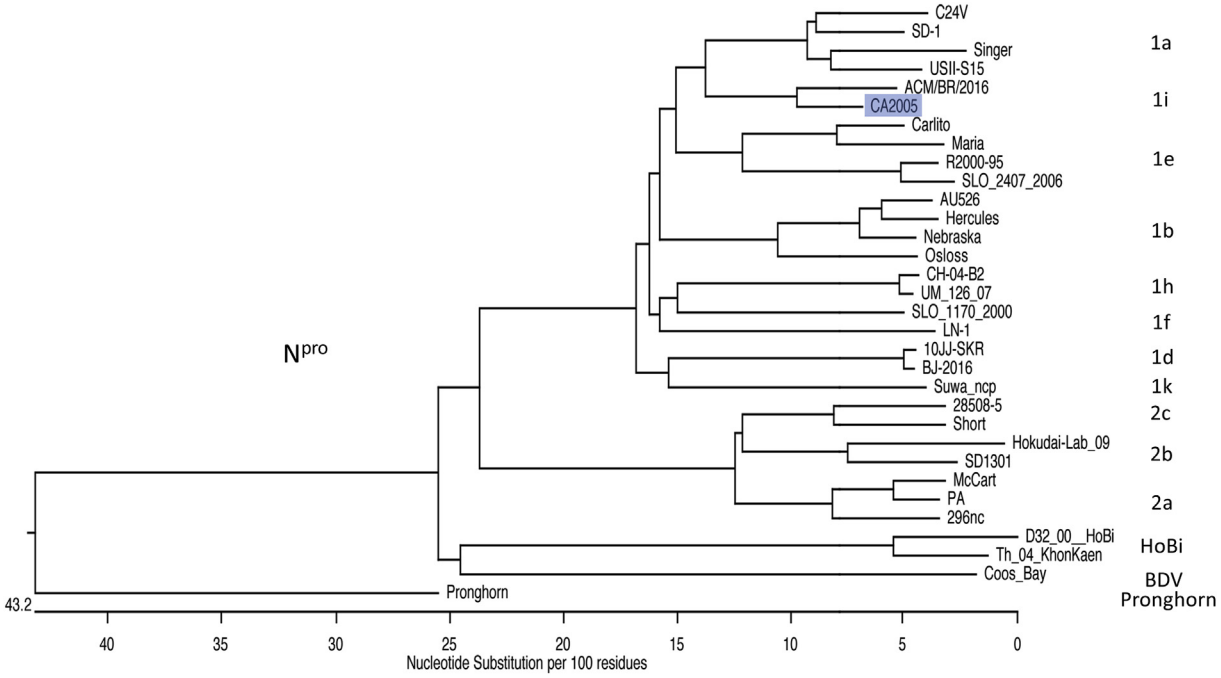

Fig. 1. Phylogenetic analysis of the sequences encoding the N^{pro} protein of pestiviruses. Phylogenetic analysis using the Npro coding sequences of 21 BVDV1, seven BVDV2, three HoBi-like viruses, one border disease virus (Coos Bay) and pronghorn virus was conducted to examine genetic relationships. The reported subgenotypes of the BVDV are shown at the right of the tree. CA2005 is highlighted in blue.

Table 2
Virus neutralization titers using BVDV1a vaccine virus-specific antisera.

	Vaccine Virus Antiserum		
	C24V	Singer	NADL
Homologous Virus	1,622[a]	5623	3236
CA2005	135	1242	135
Fold Decrease (log2)	12 (3.6)	4.5 (2.15)	24 (4.6)

[a] Reciprocal of the.

and similar viruses in vaccinated cattle.

Bovine fetal turbinate (Btu) cells were infected with CA2005 to examine the cytopathic effect (CPE) that was produced by replication in these cells. Btu cells were seeded in 96 well plates at a density of 2×10^5 cells/ml in MEM supplemented with L-glutamine and containing 10% fetal bovine serum that was tested and shown to be free of both BVDV and BVDV antibody. The cells were infected with CA2005 at a multiplicity of infection of 0.5. The plates were incubated in a humidified incubator with a 5% CO_2 atmosphere up to 96 h post-infection (PI). Fig. 2 shows the results of incubation of the CA2005-infected cells. By 48 h PI, CPE was becoming evident (Fig. 2b), with no rounding or detachment of cells visible in the 48 non-infected control cells (Fig. 2a). At 96 h PI, most of the Btu cells were rounded and detached from the

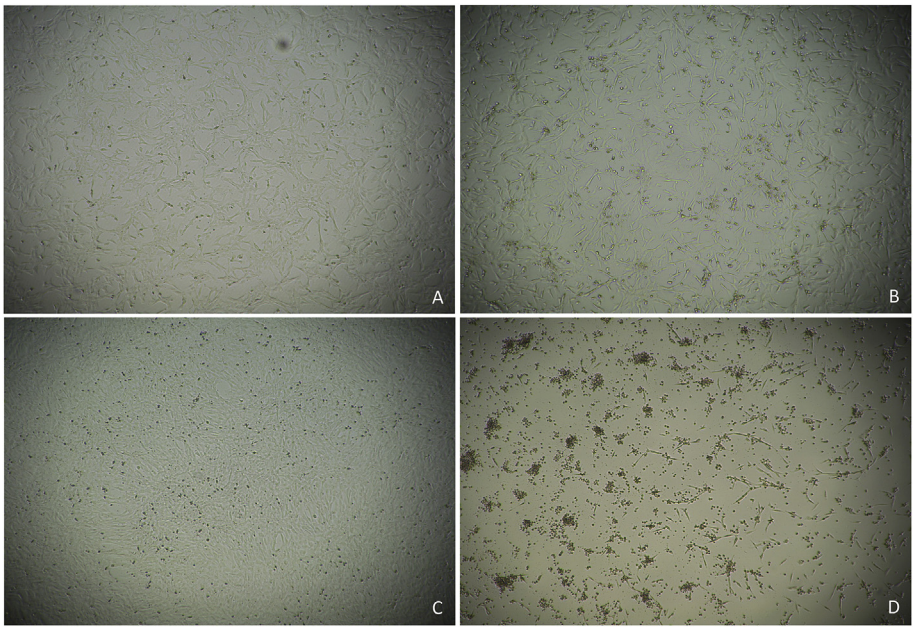

Fig. 2. Cytopathic effect in primary bovine turbinate (Btu) cells following infection with CA2005. Cells were non-infected or infected with CA2005 and observed at times post-infection for development of cytopathic effect (CPE). Fig. 3a: Non-infected Btu cells at 48 h; 3b: Btu cells infected with CA2005 at 48 h post-infection; 3c: non-infected Btu cells at 96 h; 3d: But cells infected with CA2005 at 96 h post-infection. Infected cells show CPE characteristic of cytopathic BVDV.

substrate in the CA2005-infected well (Fig. 2d) while the 96 h control well contained an intact monolayer (Fig. 2c). This demonstrated that CA2005 produced CPE similar to most BVDV1 cytopathic strains and within a similar time frame.

This report is the first description of a cytopathic BVDV1i as well as a cytopathic BVDV1 that belonged to neither the 1a or 1b subgenotypes. CA2005 clearly segregated separately from the BVDV1a and 1b and with a BVDV1i (Fig. 1). The cytopathogenicity of CA2005 was conferred as in other BVDV1 by duplication of the NS3 coding sequences and the presence of a cellular ubiquitin mRNA insertion. Antigenic characterization of CA2005 with antiserum raised against BVDV1a vaccine strains showed that there may be reason for concern for infection of cattle by non-1a and 1b viruses. There may be an increased possibility of infection by the non-1a, non-1b BVDV1 strains like CA2005 given the weak cross-neutralization observed with the vaccine virus antisera. Based on this, some vaccines may not protect well against non-1a, non-1b viruses should they appear in cattle populations. Globalization of vaccine companies has led to vaccines being developed using strains isolated in the US and being marketed worldwide rather than regional vaccines developed using stains isolated from the geographic regions in which the vaccines are to be used. Most modified live vaccines contain cytopathic viruses, as cytopathic viruses cannot establish persistent infections. One of the problems with developing vaccines based on the prevalent BVDV subgenotypes in a region is the paucity of cytopathic viruses outside of the BVDV1a, BVDV1b and BVDV2a subgenotypes. The discovery of a stable BVDV1i cytopathic virus will ease antigenic cross-reactivity studies and raises the possibility of producing multivalent vaccines that offer a broader antigenic profile. However, experiments addressing these observations still remain to be conducted.

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