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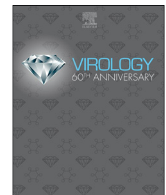
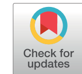


# *In vitro* method to evaluate virus competition between BVDV-1 and BVDV-2 strains using the PrimeFlow RNA assay



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ABSTRACT

Bovine viral diarrhea viruses (BVDV), segregated in BVDV-1 and BVDV-2 species, lead to substantial economic losses to the cattle industry worldwide. It has been hypothesized that there could be differences in level of replication, pathogenesis and tissue tropism between BVDV-1 and BVDV-2 strains. Thus, this study developed an *in vitro* method to evaluate virus competition between BVDV-1 and BVDV-2 strains. To this end the competitive dynamics of BVDV-1a, BVDV-1b, and BVDV-2a strains in cell cultures was evaluated by a PrimeFlow RNA assay. Similar results were observed in this study, as was observed in an earlier *in vivo* transmission study. Competitive exclusion was observed as the BVDV-2a strains dominated and excluded the BVDV-1a and BVDV-1b strains. The *in vitro* model developed can be used to identify viral variations that result in differences in frequency of subgenotypes detected in the field, vaccine failure, pathogenesis, and strain dependent variation in immune responses.

## 1. Introduction

Ruminant pestiviruses are globally-distributed pathogens responsible for a broad range of clinical presentations, which range from mild to severe and may affect the respiratory, digestive and/or the reproductive system (MacLachlan and Dubovi, 2011). Bovine viral diarrhea virus-1 (BVDV-1) and BVDV-2 are grouped in two different species, Pestivirus A and B, respectively, with multiple subgenotypes and belong to the genus *Pestivirus*, family *Flaviviridae*. Pestiviruses are single-stranded, positive-sense RNA viruses. The viral genome is approximately 12.3 Kb long and contains a unique open reading (ORF) frame that is flanked by 5′ and 3’ untranslated regions (UTRs). The ORF encodes a long polyprotein that is processed into the following polypeptides: Npro-C-Erns-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B (Smith et al., 2017).

Pestiviruses are notable for their broad host range and cell tropism and the presence of two biotypes, noncytopathic (ncp) and cytopathic (cp). In addition, clinical presentations include a wide spectrum of virulence including acute and persistent infections invariably accompanied by immunosuppressive effects. These viruses are also frequent contaminants of biological products and vaccines (Barkema et al., 2001; Fulton et al., 2003; Palomares et al., 2013; Schweizer and Peterhans, 2014; Kelling et al., 2002). Based on genetic variability, BVDV-1 and BVDV-2 can be further segregated into several subgenotypes, BVDV-1a-u and BVDV-2a-c (Yeşilbağ et al., 2017). Current licensed vaccines in the US only contain BVDV-1a and BVDV-2a strains (Fulton, 2015), but the most recent surveillance would suggest that BVDV-1b strains are the most prevalent in the US (Fulton et al., 2006; Workman et al., 2016).

While it is possible that the emergence and increased prevalence of BVDV antigenically and genetically different from vaccine strains is driven by vaccination escapes, other factors such as virulence, contact between cohorts and viral fitness could also contribute to emergence and increased prevalence of a particular strain or subgroup. Previous research has reported predominance of viral strains in simultaneous experimental challenge and *in vivo* transmission studies (Brock and Chase, 2000; Frey et al., 2002; Makoschey and Janssen, 2011; Peddireddi et al., 2018; Walz et al., 2018; Zimmer et al., 2002). Most recently two BVDV exposure studies using simultaneous exposure to PI's infected with either BVDV 1a, 1b or 2a subgenotypes reported that the 2a strain was most frequently isolated from exposed animals (Peddireddi et al., 2018; Walz et al., 2018).

Viral fitness is used to describe the ability of a virus to replicate in a

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given environment and to be transmitted to new hosts to survive. Differences in viral fitness may impact the prevalence and emergence of different BVDV species and subgenotypes. There are a variety of methods and parameters that can be used to assess strains that have the potential for increased fitness. One method that is commonly used as a measure of viral fitness is an *in vitro* viral competition assay. This type of assay is accomplished by competition experiments, in which cells or hosts are infected with two or more viral isolates. Viral competition may be evaluated at one time point or at multiple time points post-infection (Domingo, 1997; Wargo and Kurath, 2012). Traditional methods that can be used to characterize virus competition include, DNA sequencing, PCR, and monoclonal antibodies. A drawback of these methods is that they provide a consensus of the overall population but cannot define viral dynamics at the single cell level. Therefore, an *in vitro* model was developed that allows comparison of infection at the single cell level for BVDV-1a, -1b and -2a strains. To accomplish this, BVDV-1a, -1b, and -2a strains isolated from persistently infected calves previously used in an *in vivo* transmission experiment (Walz et al., 2018), were used in a novel *in vitro* competition assay. This assay was based on a flow cytometry technique that allows a multiplex detection of RNA at cellular level using in situ hybridization-based branched DNA amplification (Falkenberg et al., 2017, 2019), as well as PCR and sequencing methods for viral detection for comparisons.

## 2. Material and methods

### 2.1. Viruses

Four non-cytopathic (ncp) BVDV strains, AU-PI-34 (BVDV-1a), AU-PI-285 (BVDV-1b), AU-PI-28 (BVDV-2a) and AU-PI-12 (BVDV-2a) (henceforth referred to as PI34, PI285, PI28 and PI12, respectively), were used in the study. The strains were isolated during an *in vivo* transmission study in which naïve pregnant cattle were exposed to PI calves infected with different BVDV strains (Walz et al., 2018). The BVDV-2a subgenotype predominated in live-born calves, accounting for 56% of the subgenotypes isolated. It is unknown why the most frequently isolated subgenotype was from the BVDV-2a PI's. Viral titers from serum and nasal swabs of all the PI calves used were similar. While more fetuses were positive for BVDV-1b isolates (5 fetuses) rather than BVDV 2a isolates (4 fetuses), the total number of aborted fetuses was minimal (11) (Walz et al., 2018). Thus, for the *in vitro* competition assay the representative strain of each subgenotype that resulted in the greatest number of PI animals, PI34 (BVDV-1a), PI285 (BVDV-1b), PI28 (BVDV-2a) were selected. Since the BVDV-2a strains predominated and also accounted for a significant portion of the aborted fetuses, the BVDV-2a strain (PI12) that generated the lowest number of BVDV2a PI calves were also selected to evaluate if this predominance was strain rather than subgenotype dependent. The approximately percent of the total number of PI progeny each PI contributed to is as follows; PI34 10%, PI285 14%, PI12 10% and PI28 34%.

All strains were recovered from the serum of the PI's used to expose pregnant cows. Genome sequences for isolated strains were determined using a sequence-independent method using the MiSeq platform to confirm their identity (Neill et al., 2014). Then, the full length sequences were assembled and analyzed using Lasergene 12 package (DNAstar, Inc., Madison, WI) and Aligner (Codoncode, Inc., Centerville, MA, USA). The GenBank accession number of the sequences are MH23114 (PI12), MH231141 (PI28), MN188073 (PI34), MN188074 (PI285). No other viral sequences than the BVDV strains mentioned above were found.

The four ncp BVDV strains were passed four times in Madin-Darby bovine kidney (MDBK) cell line prior to use in the assay, and titrated using an immunoperoxidase staining with the monoclonal antibody N2 (Bauermann et al., 2012; Ridpath and Neill, 2000).

### 2.2. Cells

MDBKs were used to propagate the viral stocks used in the study, while two other cell types were used for the virus competition assay. The two other cell types used consisted of a primary bovine turbinate epithelial adherent cell culture (BTu) and a bovine lymphoma B cell suspension cell line (BL-3). These cell types were chosen to represent epithelial and lymphoid cells, which are the primary sites of replication of the BVDV in acute infection (Liebler-Tenorio et al., 2003). The BL-3 cell line (CRL-8037) received from the American Type Culture Collection (ATCC) was contaminated with BVDV. Subsequently, a BVDV free NADC-BL-3-SF cell line was derived from the contaminated line by limiting dilution (Falkenberg et al., 2017; Ridpath et al., 2006). The BTu cells were primary cells derived from bovine fetal tissues in our lab.

The NADC-BL3-SF (BL-3) cell line was maintained in RPMI-1640 medium, MDBK and BTu cells in MEM medium (Sigma Aldrich), both media supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (PAA Laboratories Inc. Ontario, Canada), L-Glutamine (Thermo Fisher Scientific, Waltham, MA) and antibiotic-antimycotic. Cells were incubated at 37 °C in a humid atmosphere with 5% $CO_2$. The cells and FBS were confirmed to be free of pestivirus RNA by RT-PCR (Bauermann et al., 2013). In addition, FBS was also tested and found to be free of pestivirus-antibodies by virus neutralization assay using the strains Singer (BVDV-1a), 296c (BVDV-2a), and Italy 1/10-1 (HoBi-like virus) (Vilcek et al., 1994).

### 2.3. Viral infection and experiment design

The competitive assay was conducted as single, dual, and triple infections in the two cell types, using the PI34 (BVDV-1a), PI285 (BVDV-1b), PI28 (BVDV-2a) and PI12 (BVDV-2a) strains, obtained and titered from passage four in MDBK as described above. The experiment was repeated three different times. The procedures used to inoculate the cells are as follows and a schematic of the experiment design can be found in Fig. 1. Approximately, $10^7$ BL-3 cells (1 flask-75 $cm^2$) were spun down (300 × *g*, 10 min), spent (conditioned) media was harvested (for later use), and cells were inoculated with each strain (single, dual, and triple infections) then placed on a rocker at 37 °C for 1 h. At the end of this time period, the cells were spun down, the supernatant discarded and a 50/50 ratio of spent/fresh media was added back to the cells (30 mL total). BTu cells ($10^6$) at ~70% confluence were inoculated with each strain (3 flasks-25 $cm^2$) and incubated at 37 °C for 1 h with rocking, followed by replacement of the inoculum with fresh media. Both cell types were incubated at 37 °C in a humid atmosphere with 5% $CO_2$ and infected at an MOI of 1.0 and 0.1 to evaluate differences that may be observed due to MOI. Absolute cell numbers for both cell types were determined by flow cytometry using counting beads (SPHERO AccuCount-Spherotech) to determine the respective MOI for each cell type and for each respective virus. In addition, both cell types were mock-infected using the respective cell media from negative control cells.

The flasks of BVDV infected cells that were not used for analysis at each respective time point (days 2, 9, and 30) were maintained as follows: the BL-3 cells (1 flask - 75 $cm^2$) were passed 1/3 twice a week and the BTu cells were trypsinized and passed 1/4 once a week (3 flasks – 25 $cm^2$) (Fig. 1).

On 2, 9, and 30 days post infection (dpi), cells were harvested (BL-3 – 10 mL from the one 75 $cm^2$ flask) or trypsinized (BTu - 2 flasks 25 $cm^2$) for evaluation of viral competition dynamics. At these time points, cells were used in the PrimeFlow RNA assay as well as, cells (~$10^5$) were collected for RNA extraction to perform RT-qPCR and RT-PCR for sequencing. After performing the PrimeFlow RNA assay, remaining cells not used for flow cytometric analysis were spun down onto glass slides for immunofluorescence (IF) microscopy.

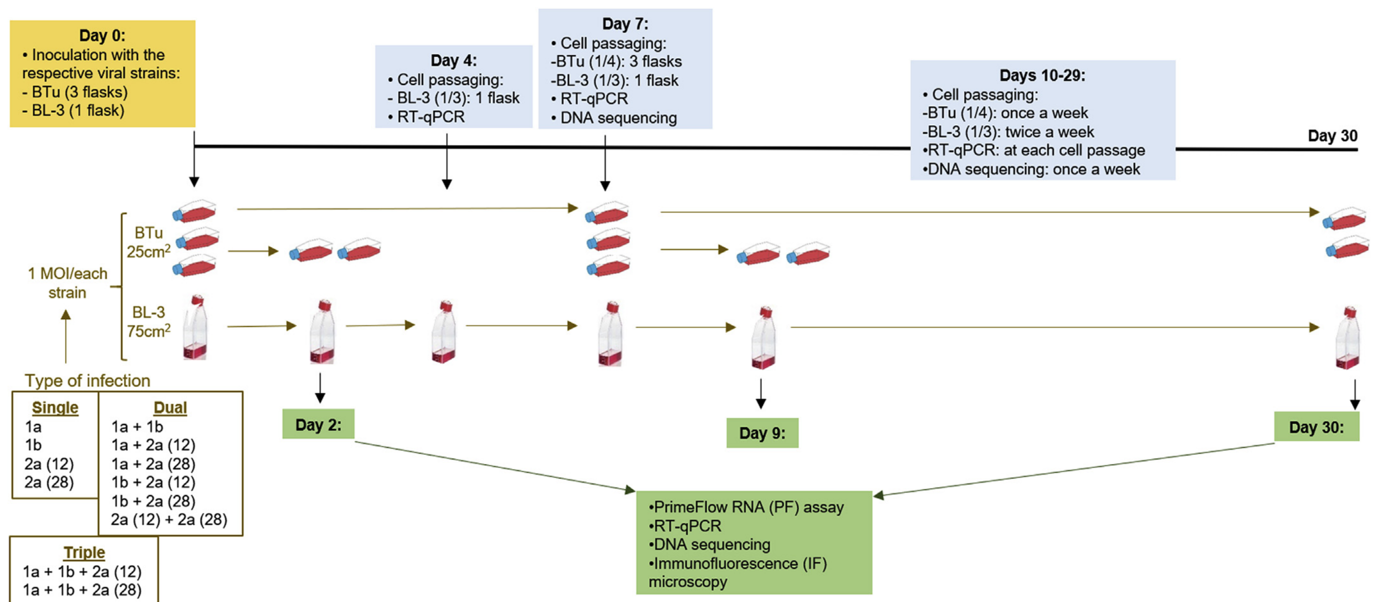


**Fig. 1.** Study workflow to evaluate competitive fitness between the strains of BVDV-1a, BVDV-1b, and BVDV-2a. The time points and details of virus inoculation, cell passages and techniques performed are depicted.

### 2.4. BVDV RNA quantification by RT-qPCR and identification by DNA sequencing

The relative RNA viral load present in the cells was determined by RT-qPCR and the predominant BVDV strain present was determined by DNA sequencing.

An aliquot of 140 μL of cell culture suspension (~$10^5$ cells) was submitted to RNA extraction using QIAcube® (Viral RNA kit) according to the manufacture's recommendations (Qiagen, Valencia, CA). Extracted RNA was used for both RT-qPCR as well as conventional RT-PCR for DNA sequencing. For virus quantification, a commercial RT-qPCR kit was used to detect a fragment of the BVDV-1 and BVDV-2 5′UTR (BVDV VetMax Gold – Thermo Fisher Scientific). Samples were analyzed using QuantStudio™ 5 System (Applied Biosystems) with the respective software. For BVDV identification, a conventional RT-PCR that targeted the 5′UTR was performed (Ridpath et al., 1994). Amplification products were purified using a QIAquick PCR Purification kit (Qiagen), according to the manufacturer's instructions, followed by DNA quantification using a Qubit fluorometer (Invitrogen Corporation, Carlsbad, CA). Both strands were sequenced in duplicate using a Big Dye Terminator cycle sequencing kit, on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were edited and aligned using Geneious software (Biomatters Inc, Newark, NJ). Final phylogenetic analyses were performed using Molecular Evolutionary Genetics Analysis software package 7 (MEGA7) (Tamura et al., 2011).

### 2.5. BVDV identification and quantification by the PrimeFlow RNA assay

The PrimeFlow RNA assay was performed to identify and quantify individual cells positive for each respective BVDV strain, using a different fluorochrome for each strain (Table 1). This technique allowed the quantification of BVDV RNA, by determination of geometric mean fluorescence intensity (gMFI). Thus, the PrimeFlow RNA assay provided the number of single, dual and triple cells positive for each strain, as well as the comparative amount of viral RNA present.

#### 2.5.1. Cell preparation

Both cell types infected with the respective viruses, virus combinations, and mock-infected cells were harvested at 2, 9, and 30 dpi as previously described (Fig. 1). Cells harvested from each respective cell type were centrifuged (300 × g, 10 min), resuspended in PBS and plated at ~ 2 × $10^6$ cells/well of a 96-well plate. Plated cell suspensions were centrifuged (300 × g for 2 min) at 4 °C, washed with PBS again to remove any residual medium and centrifuged prior to further use in the assay. Subsequently, to identify and select live cells, plated cells were stained with fixable viability dye eFluor 450 (eBioscience, San Diego, CA) as described by the manufacturer, which was followed by two wash steps with stain buffer per the manufacturer's recommendation (BD Biosciences, San Jose, CA).

**Table 1**
PrimeFlow target probe types.

| Strain | Subgenotype | Fluorochrome | Excitation (max) | Emission (max) |
|---|---|---|---|---|
| PI34 | BVDV-1a | Type 1 probe (AF647)® | 647 nm | 668 nm |
| PI285 | BVDV-1b | Type 4 probe (AF488)® | 488 nm | 519 nm |
| PI12 | BVDV-2a | Type 6 probe (AF750)® | 749 nm | 775 nm |
| PI28 | BVDV-2a | Type 6 probe (AF750), | 749 nm | 775 nm |
| | | Type 1 probe (AF647)* | 647 nm | 668 nm |

*probe used for the PI28 strain in dual infections that contained the PI12 strain, both BVDV-2a.

#### 2.5.2. The PrimeFlow RNA assay

After the live/dead staining, the 96-well plates were submitted to the PrimeFlow RNA assay, which was performed according to the manufacturer's instructions (Thermo Fischer Scientific, Carlsbad, CA). The target probes corresponding to 20 gene-specific oligonucleotide (RNA) probes, were designed based on the sequence of the genomic region coding for $N^{pro}$-C-$E^{rns}$ (~1500 nt) of each strain. Probes were conjugated to a specific fluorescent dye to specifically detect the respective BVDV strain (Table 1). Probe pairs were designed by Thermo Fisher Scientific, and procedures used for the design and generation of the probes as well as the probe sequences are considered proprietary by the company (Thermo Fischer Scientific, Carlsbad, CA).

Multi-color flow cytometric analysis was performed using a BD LSRII flow cytometer (BD Biosciences). Cells were visualized in forward (FSC) and side scatter (SSC) and single cells were gated and dead cells were eliminated based on eFluor 450 dye staining. The eFluor 450 dye was excited at 405 nm laser line (violet laser) and the emission signal was detected using a 450/50 nm band-pass filter.

Single BVDV infected cells and mock-infected cells were used to set the gates and as positive and negative controls, respectively. Fluorescence-minus-one controls (lack of one target probe) and no-probe controls (lack of target probe addition), were included to set up the gates for positive cells and compensation for each fluorochrome/channel. Approximately 20,000 events per gate were acquired using LSR II equipped with violet (405 nm), blue (488 nm), and red (633 nm) lasers and data analysis was performed using FlowJo software (FlowJo LLC). In addition, probe specificity controls were employed that consisted of adding all probes to single infected cells to verify the cross-reactivity between them, or any background. That is, all probes were included, excluding the probe to detect the single virus used to infect the cells to ensure no non-specific binding was observed with any other probes.

The gMFI, expressed on the log scale, was calculated for the RNA positive cells for each strain. The gMFI was compared within each strain, over time and between single, dual or triple infections. Comparisons could only be made for each respective fluorochrome within each strain since as the fluorescent intensity varied between each fluorochrome.

### 2.6. BVDV identification by immunofluorescence (IF) microscopy

Cells from the PrimeFlow RNA assay were subjected to the IF to verify the results observed in the PrimeFlow RNA assay, and to assess the cellular localization of each respective strain. The cell suspension (50 µL) was spun down onto glass slides using a Cytospin cytocentrifuge (Thermo Shandon Cytospin 3) at $10.16 \times g$ for 3 min and coverslips were mounted using ProLong Gold antifade mount medium with DAPI (ThermoFisher, Carlsbad, CA). Fluorescent imaging was performed on a Nikon A1R + Confocal System microscope (Nikon Instruments, Melville, NY), with three fluorescent channels (AF488, AF647, AF750) using a 40× oil-immersion objective. NIS-Elements Advanced Research software was used for image analysis and metadata files were saved as proprietary Nikon ND files.

## 3. Results

The competitive assay was conducted as single, dual, and triple infections in two cell types (BL-3 and BTu). Four BVDV strains were used to infect the cells: one strain of BVDV-1a (PI34), one of BVDV-1b (PI285) and two strains of BVDV-2a (PI12 and PI28). Cells were infected using equal MOIs of each virus, harvested at three time points (2, 9, and 30 dpi) and submitted to the RT-qPCR for virus quantification, DNA sequencing, PrimeFlow RNA assay and IF. Each experiment was conducted in triplicate and analogous results were observed between each of the replicates. As there was no difference in trends or frequency of virus positive cells between the replicates, the data from one replicate is reported. Further, an MOI of 1 and 0.1 were evaluated for differences in viral dynamics due to MOI, but no difference was observed as the same trends were observed for each respective MOI. Therefore, only results using an MOI of 1 will be reported.

### 3.1. BVDV RNA quantification by RT-qPCR

Differences in virus quantification within the cell types, over time and between the types of infection were analyzed using an RT-qPCR assay that did not differentiate between strains. The cycle threshold (Ct) values of single, dual or triple infections in BL-3 cells at day 2 ranged from 19.9 to 23.1. On day 2, all types of infection containing strain PI12 (BVDV-2a) had higher Ct values (22.6–23.1) than the other types of infection (19.9–21.4; Table 2). A gradual increase in Ct values was observed on day 9 (23.1–24.6) and again on day 30 (24.6–27.7). However, at day 30, single infections had higher Ct values (26.8–27.7) than dual and triple infections (24.6–25.2; Table 2).

The Ct values observed in BTu cells at day 2 infection were very similar among the single, dual and triple infections, ranging from 15.0 to 16.1, but these values were lower than Ct values of BL-3 cells (19.9–23.1). Similar to BL-3 cells, a gradual increased in Ct values was observed on day 9 (23.1–24.6) and again on day 30 (24.6–27.7). The single infections with 1a-strain and 1b-strain had the highest Ct values (23.2 and 25) among all types of infection at day 9, which decreased at day 30 (20.1–20.8; Table 3).

Collectively, these observations suggest that these BVDV strains have a higher replication rate in BTu cells when compared to BL-3 cells. In both cell types, the Ct values were lower on day 2 when compared to day 9 (Tables 2 and 3). From day 9 to day 30 a slight Ct increase was observed in all types of infection in BL-3 cells and in the majority of the Btu cells (Tables 2 and 3). All mock-infected samples harvested from the two cell types in all time points had Ct values higher than 36, which was considered negative.

**Table 2**
RT-qPCR Ct values and DNA sequencing identification in BL-3 cells.

| BL-3 | Day 2 | | Day 9 | | Day 30 | |
|---|---|---|---|---|---|---|
| *Virus used for infection | RT-qPCR Ct value | DNA sequencing | RT-qPCR Ct value | DNA sequencing | RT-qPCR Ct value | DNA sequencing |
| 1a | 20.8 | 1a | 23.1 | 1a | 27.4 | 1a |
| 1b | 19.9 | 1b | 24.3 | 1b | 27.7 | 1b |
| 2a (12) | 23.1 | 2a | 24 | 2a | NT | 2a |
| 2a (28) | 20.5 | 2a | 24.4 | 2a | 26.8 | 2a |
| 1a/1b | 20.3 | UND | 24 | UND | 24.6 | 1b |
| 1a/2a (12) | 22.6 | 2a | 23.9 | 2a | NT | NT |
| 1a/2a (28) | 20.9 | 2a | 24.6 | 2a | 24.6 | 2a |
| 1b/2a (12) | 22.7 | 2a | 23.9 | 2a | NT | NT |
| 1b/2a (28) | 21.1 | 2a | 24.3 | 2a | 24.7 | 2a |
| 2a (12)/2a (28) | 21.4 | 2a | 23.9 | 2a | NT | NT |
| 1a/1b/2a (12) | 22.9 | 2a | 24 | 2a | NT | NT |
| 1a/1b/2a (28) | 20.4 | 2a | 24.2 | 2a | 25.2 | 2a |

*Bovine viral diarrhea virus strains; PI34 (1a), PI285 (1b), PI28 and PI12 (2a).
NT- Not tested.
UND – undetermined.

### 3.2. BVDV identification by DNA sequencing

While RT-qPCR confirmed the presence as well as the relative amount of BVDV in each cell type at three different time points, this assay could not be used to determine the strain(s) of BVDV prevalent in dual and triple infected cells since universal forward and reverse BVDV primers were used. Therefore, DNA sequencing was performed (with amplified DNA products) to determine which BVDV strains were present in the cells at the respective time points. While all viruses could be identified for single infections at all time points, infections with multiple viruses either provided sequence that appeared to be mixed due to multiple peaks, or identified the more frequent sequence if strains were present in unequal amounts.

In the BL-3 cells, from day 2 onward, if a BVDV-2 strain was used to infect the cells, only the BVDV-2 strains were identified by sequencing (Table 2). No clear sequence was obtained in the dual infection with 1a and 1b-strains at day 2 and 9, but the 1b-strain was identified at day 30 (Table 2).

Similar results were also observed in the BTu cells at day 30 (Table 3). However, on day 2, no unique BVDV sequence was observed in the following three infection types: (1a/1b), [2a (PI12)/2a (PI28)], [1a/1b/2a (PI28)] (Table 3). In contrast to dual and triple infections including 2a-strains in BL-3 cells, in BTu infections, the sequence of either the 1a or 1b-strain could be identified in dual and triple infections on day 2 (Table 3). By day 9, the 2a-strains were identified by

**Table 3**
RT-qPCR Ct values and DNA sequencing identification in BTu cells.

| *BTu | Day 2 | | Day 9 | | Day 30 | |
|---|---|---|---|---|---|---|
| Virus used of infection | RT-qPCR Ct value | DNA sequencing | RT-qPCR Ct value | DNA sequencing | RT-qPCR Ct value | DNA sequencing |
| 1a | 15 | 1a | 23.2 | 1a | 20.1 | 1a |
| 1b | 15.3 | 1b | 25 | 1b | 20.8 | 1b |
| 2a (12) | 15.6 | 2a | 19.9 | 2a | 21.5 | 2a |
| 2a (28) | 15.5 | 2a | 23.2 | 2a | 22.5 | 2a |
| 1a/1b | 15.2 | 1a | 20.6 | 1a | 20.9 | 1b |
| 1a/2a (12) | 15.4 | 1a | 20 | UND | 21 | 2a |
| 1a/2a (28) | 15 | 1a | 23.6 | 2a | 22.7 | 2a |
| 1b/2a (12) | 15.7 | 1b | 20.6 | 2a | 21.6 | 2a |
| 1b/2a (28) | 15.1 | UND | 19.7 | 2a | 22.5 | 2a |
| 2a (12)/2a (28) | 15.2 | UND | 20.3 | 2a | 22.8 | 2a |
| 1a/1b/2a (12) | 16.1 | 1a | 19.6 | 2a | 21.3 | 2a |
| 1a/1b/2a (28) | 15.4 | UND | 20.2 | 2a | 21.5 | 2a |

*Bovine viral diarrhea virus strains; PI34 (1a), PI285 (1b), PI28 and PI12 (2a).
UND – undetermined.

sequencing in the dual and triple infected cells if a 2a-strain was used to infect BTu cells and this continued through day 30 (Table 3). The 1a-strain was identified on day 2 and day 9 in the infection with 1a/1b dual infection, but the 1b-strain was identified on day 30 (Table 3).

### 3.3. BVDV identification and quantification by the PrimeFlow RNA assay

In contrast to PCR amplification followed by DNA sequencing, the PrimeFlow RNA assay provided simultaneous identification of the strains and quantification of prevalence based on the percentage of infected and co-infected cells. Moreover this assay allowed comparison of BVDV RNA load based on the gMFI.

#### 3.3.1. BL-3 cells

On day 2, the total frequency of virus positive cells in single infections ranged from 43.8 to 96.4% (Table 4). The total frequency of virus positive cells for dual and triple infections ranged between 39.1 and 94.8%, with the range of percent infected with each respective strain from 0 to 94.4% (Table 4). Within the dual and triple infections, the frequency of virus positive cells with two or more viruses ranged from 0.8 to 33.1% (Table 4).

On day 9, the total frequency of virus positive cells from single infections ranged from 12.1 to 31.1% (Table 4). The total frequency of virus positive cells for dual and triple infections ranged between 12.1 and 33%, with the range of percent infected with each respective strain from 0 to 26.8% (Table 4). Within the dual and triple infections, the frequency of virus positive cells with two or more viruses ranged from 0 to 3.6% (Table 4). The total percentage of virus positive cells decreased from day 2 to day 9, and the gMFI as a relative measure of the amount of virus in the cells also decreased substantially from day 2 to day 9 (Table 4).

On day 30, the total frequency of virus positive cells from single infections ranged from 15% to 40.5% (Table 4). The total frequency of virus positive cells for dual and triple infections ranged between 15.4% and 36.5%, with the range of percent virus positive cells with each respective strain from 0 to 34.5% (Table 4). Within the dual and triple infections, the frequency of virus positive cells with two or more viruses ranged from 0% to 1% (Table 4). The total percentage of virus positive cells remained similar from day 9 to day 30, and the gMFI was also similar on day 9 to day 30 (Table 4).

The lowest frequency of positive cells following inoculation was observed with the BVDV-2a PI12-strain. However, while this strain had the lowest frequency of positive cells, the 2a-strains (PI28 and PI12) began to outcompete 1a and 1b strains early in dual and triple infections, at day 2, with the PI28 (2a)-strain being the most competitive. However, when the cells were co-infected with both two 2a-strains, the PI28-strain outcompeted and virtually eliminated the PI12-strain at day 9 (Table 4).

Since the two 2a-strains had a similar behavior in dual and triple infections with the 1a and 1b-strains, only the infections with the PI28 (2a)-strain were maintained until the end of the study.

The dual infection 1a/1b showed more dual positive cells at the three-time points, with the maximum of 33.1% at day 2 (Table 4). The PI34 (1a)-strain and PI285 (1b)-strain were those that coexisted best in dual infection. Moreover, this type of infection had one of the highest total percentage of virus positive cells at the three time points, followed by their single infections and the dual infection with the two 2a-strains PI12 and PI28. The similar frequency of 1a and 1b virus positive cells in the dual 1a/1b infection remained until day 9; however, afterwards the 1b-strain predominated at day 30.

#### 3.3.2. BTu cells

On day 2, the total frequency of virus positive cells from single infections was 99.9–100% (Table 5). The total frequency of virus positive cells for dual and triple infections ranged between 99.4 and 99.9%, with the range of virus positive cells with each respective strain from 42.7 to 99.2% (Table 5). Within the dual and triple infections, the frequency of viral positive cells with two or three viruses ranged from 29.4 to 89.5% (Table 5). The frequency of BVDV positive cells in dual and triple infections at day 2 were higher in BTu cells compared to BL-3 cells.

On day 9, the total frequency of virus positive cells ranged from 41.6 to 60.3% (Table 5). The total frequency of virus positive cells for dual and triple infections ranged between 51.7 and 60.9%, with the range of percent virus positive with each respective strain from 11.9 to 52.5% (Table 5). Within the dual and triple infections, the frequency of virus positive cells with two or more viruses ranged from 0.7 to 7.8% (Table 5). As observed in the BL-3 cells, the total percentage of infected cells decreased from day 2 to day 9, and similarly the gMFI also decreased substantially from day 2 to day 9 (Table 5).

On day 30, the total frequency of virus positive cells from single infections ranged from 58.5 to 69.3% (Table 5). The total frequency of virus positive cells for dual and triple infections ranged between 56.2 and 65.7%, with the range of percent virus positive cells with each respective strain from 0 to 63.7% (Table 5). Within the dual and triple infections, the frequency of virus positive cells with two or more viruses ranged from 0 to 11.6% (Table 5). While total percentage of virus positive cells remained similar from day 9 to day 30, and the gMFI was also similar on day 9 to day 30, by day 30 2a-strains (PI12 or PI28) outcompeted the BVDV-1 isolates (Table 5). This was similar to the trend seen in dual and triple infections in BL-3 cells. Although DNA sequencing detected 2a sequence at day 9 in triple infected BTu cells

**Table 4**
Percentage of single, dual, and triple positive cells and gMFI of positive BL-3 cells.

| BL-3 | Day 2 | | | Day 9 | | | Day 30 | | |
|---|---|---|---|---|---|---|---|---|---|
| *Virus used for infection | gMFI per strain | % positive cells (total and per strain**) | % co-positive cells (total and per combination**) | gMFI per strain | % positive cells (total and per strain**) | % co-positive cells (total and per combination**) | gMFI per strain | % positive cells (total and per strain*) | % co-positive cells (total and per combination**) |
| 1a | 24991 | 95 | 0 | 14882 | 29.8 | 0 | 8394 | 40.5 | 0 |
| 1b | 14221 | 96.4 | 0 | 8991 | 31.1 | 0 | 9186 | 30.5 | 0 |
| 2a (12) | 766 | 43.8 | 0 | 743 | 15.1 | 0 | NT | NT | NT |
| 2a (28) | 1236 | 85.2 | 0 | 729 | 12.1 | 0 | 735 | 15 | 0 |
| 1a/1b | 17372/ 9923 | 93.6 = 65.8/60.9 | 33.1 | 15121/ 8633 | 33 = 20.7/15.9 | 3.6 | 9400/ 9387 | 36.5 = 3.0/34.5 | 1 |
| 1a/2a (12) | 5490/694 | 48.8 = 24.5/32.5 | 8.2 | na/701 | 0/13.6 | 0 | NT | NT | NT |
| 1a/2a (28) | 8583/1175 | 83.5 = 1.2/83.2 | 0.9 | na/759 | 0/12.1 | 0 | na/753 | 0/15.8 | 0 |
| 1b/2a (12) | 9689/716 | 39.1 = 1.7/38.2 | 0.8 | na/710 | 0/13.9 | 0 | NT | NT | NT |
| 1b/2a (28) | 6205/1173 | 83.5 = 5.4/82.7 | 4.6 | 7302/788 | 14.6 = 1.2/13.6 | 0.2 | na/738 | 0/15.4 | 0 |
| 2a (12)/2a (28) | 597/8287 | 94.8 = 4.5/94.4 | 4.1 | 701/4662 | 27.4 = 0.7/26.8 | 0.1 | NT | NT | NT |
| 1a/1b/2a (12) | 5479/na/ 727 | 52.2 = 27.2/0/35 | 10 (1a2a) | na/na/791 | 0/0/17.3 | 0 | NT | NT | NT |
| 1a/1b/2a (28) | na/6125/ 1155 | 79.9 = 0/4.5/78.8 | 3.7 (1b2a) | na/6874/ 816 | 17 = 0/2.4/15.2 | 0.6 (1b2a) | na/na/ 753 | 0/0/16.6 | 0 |

*Bovine viral diarrhea virus strains; PI34 (1a), PI285 (1b), PI28 and PI12 (2a).
NT- Not tested.
na – not applicable, when the % of positive cells is 0.
** It refers to the total % of positive cells by a particular strain or combination of strains. However, it does not mean that % of cells are exclusively positive for the particular strains.

[1a/1b/2a (PI12)] (Table 3), results from the PrimeFlow RNA assay revealed that the frequency of 1a strain infected cells was highest (33.2%) as compared to 1b (17.6) and 2a-PI12 (18.1%) in triple infected BTu cells (Table 5). Similar difference was also observed for 1a/1b infected BTu cells on day 30, with the 1a-strain having a greater number of virus positive cells, but the 1b strain was detected by sequencing.

While differences in viral strain replication were observed in both cell types, these differences were evident earlier in the BL-3 cells (day 9) compared to BTu cells (day 30). Specifically, the PI28 (2a)-strain excluded 1a-strain and 1b-strain in dual and triple infections. While the PI12 (2a)-strain did not eliminate other BVDV strains, at 30 days, there were a greater number of cells positive for the PI12 (2a)-strain as compared to the 1a-strain and 1b-strain, although there were a small percentage of cells positive for the 1a-strain and the 1b-strain. The PI28 (2a)-strain also outcompeted the PI12 (2a)-strain in dual infections (Table 5).

As in the BL-3 cells, the dual infection 1a/1b was responsible for the greatest percentage of dual positive cells. Whereas the 1b-strain outcompeted the 1a-strain in the BL-3 cells, in the BTu's, a similar number of cells were virus positive for each respective strain.

### 3.4. *Immunofluorescence (IF) microscopy*

IF was used to corroborate the results observed using the PrimeFlow RNA assay. IF provided the opportunity to discern if viral strains were co-localized in cells that were positive for both or all viruses. Images obtained for IF did not suggest that BVDV strains were co-localized within the same area of the cytoplasm. The BVDV straining was dispersed throughout the cytoplasm of cell rather than concentrated in one particular area. No fluorescence signal was detected in negative (mock-infected) cells. Given that the IF images were unremarkable, data is not shown.

## 4. Discussion

The competitive dynamics of BVDV-1a, BVDV-1b, and BVDV-2a strains were evaluated in two cell types using multiple methods of viral detection/characterization. The same strains had been previously been used in an *in vivo* transmission study (Walz et al., 2018). Similar results were observed in both studies, regardless of the cell type or method of viral characterization used, the 2a strains outcompeted 1a and 1b-strains. Furthermore, in *in vitro* studies, a 2a-strain (PI28) that had a high rate of transmission *in vivo*, outcompeted a 2a-strain (PI12) that had also a lower transmission rate *in vivo*.

Multiple cell lines were chosen to represent an important site for replication of the virus as well as cells that were important in establishment of initial infection in the respiratory tract (BTu) followed by systemic viral replication in lymphoid tissues (BL-3). Multiple methods of viral detection and characterization were used to represent traditional methods, RT-qPCR for quantification and DNA sequencing for characterization of the specific viral strain, and the PrimeFlow RNA assay as a novel method that combines both the identification of the specific strain at the cellular level but also provides the ability to quantify the virus.

RT-qPCR is frequently used to compare viral loads in cultures and tissues. RT-qPCR provides a general characterization of total amount of virus but is unable to determine the number or ratio of individual cells that are virus positive. However, the Ct values observed between cell types and over time corroborated the results observed with the PrimeFlow RNA assay. RT-qPCR values were lower, indicating more virus, in the BTu cell than in BL3 cell type, which was in agreement with frequency of virus positive cells in the PrimeFlow RNA assay. Regarding the difference between the time points, samples from day 2 resulted in the lowest Ct values in both cell types and the greatest number of virus positive cells. Therefore, the general trends associated

**Table 5**
Percentage of single, dual, and triple positive cells and gMFI of positive BTu cells.

| BTu | Day 2 | | | Day 9 | | | Day 30 | | |
|---|---|---|---|---|---|---|---|---|---|
| *Virus used for infection | gMFI per strain | % positive cells (total and per strain**) | % co-positive cells (total and per combination**) | gMFI per strain | % positive cells (total and per strain**) | % co-positive cells (total and per combination**) | gMFI per strain | % positive cells (total and per strain**) | % co-positive cells (total and per combination**) |
| 1a | 63489 | 100 | 0 | 13523 | 60.3 | 0 | 16289 | 69.3 | 0 |
| 1b | 111907 | 99.9 | 0 | 20889 | 41.6 | 0 | 25817 | 56.5 | 0 |
| 2a (12) | 6450 | 100 | 0 | 1053 | 59.1 | 0 | 1188 | 64.8 | 0 |
| 2a (28) | 6661 | 99.9 | 0 | 1177 | 57 | 0 | 1287 | 58.5 | 0 |
| 1a/1b | 48550/ 36268 | 99.7 = 99.2/90 | 89.5 | 12928/ 21100 | 55.2 = 41.3/21.7 | 7.8 | 14531/ 23165 | 62.3 = 41.1/32.8 | 11.6 |
| 1a/2a (12) | 55481/ 3520 | 99.9 = 94.9/53.9 | 48.9 | 12744/ 1082 | 59.5 = 40.9/25.5 | 6.9 | 13401/ 1191 | 62.3 = 11/54.3 | 3 |
| 1a/2a (28) | 48419/ 3586 | 99.9 = 95.4/47.6 | 43.1 | 12336/ 1162 | 55.8 = 34/28.6 | 6.8 | na/1293 | 0/61.1 | 0 |
| 1b/2a (12) | 57491/ 1798 | 99.7 = 95.7/63.3 | 59.3 | 20859/ 1035 | 52.1 = 31/26.8 | 5.7 | 21718/ 1096 | 56.2 = 7.3/50.9 | 2 |
| 1b/2a (28) | 49551/ 1453 | 99.4 = 87.2/65.4 | 53.2 | 19348/ 1131 | 51.7 = 15.4/41 | 4.7 | na/1263 | 0/58.9 | 0 |
| 2a (12)/2a (28) | 2413/ 56699 | 99.9 = 75.5/89.5 | 65.1 | 922/14516 | 60.9 = 16.2/52.5 | 7.8 | 724/16247 | 65.7 = 5.8/63.7 | 3.8 |
| 1a/1b/2a (12) | 43260/ 30818/ 2956 | 99.9 = 95.9/76.3/42.7 | 85.6 = 74.3 (1a1b), 39.7 (1a2a), 30.4 (1b2a), 29.4 (1a1b2a) | 12331/ 20695/ 1057 | 59.8 = 33.2/17.6/18.1 | 10.4 = 4.2 (1a2a), 0.7 (1a1b2a), 5.4 (1a1b), 2.2 (1b2a) | 13789/ 22857/ 1216 | 62 = 8.3/8.4/50.4 | 5.1 = 2.3 (1a2a), 2.3 (1b2a), 0.9 (1a1b), 0.2 (1a1b2a) |
| 1a/1b/2a (28) | 32167/ 32805/ 3418 | 99.9 = 90.4/73.1/53.2 | 84.4 = 45.5 (1a2a), 32.4 (1a1b2a), 68.5 (1a1b), 35.2 (1b2a) | 11340/ 19886/ 1164 | 52.8 = 17.9/11.9/34.3 | 8.2 = 4.5 (1a2a), 0.7 (1a1b2a), 2.3 (1a1b), 2.8 (1b2a) | na/na/ 1294 | 0/0/58.9 | 0 |

*Bovine viral diarrhea virus strains; PI34 (1a), PI285 (1b), PI28 and PI12 (2a).
na – not applicable, when the % of infected cells is 0.
** Refers to the total % of infected cells by a particular strain or combination of strains. However, it does not mean that % of cells are exclusively infected by the particular strains.

with amount of virus was similar regardless of the method used.

In this study, DNA sequencing identified the sequence of the most abundant strain in a mixed infection. If strains were present in similar amounts, sequence results could not be used to differentiate which strains were present. In contrast, the PrimeFlow RNA assay was able to identify the frequency of cells that were positive for each respective virus at each time point whether by single, dual or triple infection at the individual cell level. Thus, the PrimeFlow was the best tool to study viral competition because incorrect assumptions could be made about the viral dynamics with regard to exclusion if DNA sequencing was the only method used to characterize the viral population. Furthermore, while both the Ct values and sequencing allowed for similar conclusions and corroborated the PrimeFlow results, neither RT-qPCR or DNA sequencing provided the opportunity to clearly define the minor virus populations. In addition, RT-qPCR and sequencing are unable to describe the percentage of infected cells that are single, dual, or triple virus positive. Thus, results from this study would suggest that the PrimeFlow RNA assay provides a better picture of viral dynamics and is the best method currently available for evaluating infections at the cellular level following exposure with multiple pathogens.

While similar trends existed between studies in BL-3 and BTu cells, competitive exclusion was observable at earlier time points in BL-3 cells. Moreover, the BL-3 cells showed a higher variability in RNA viral load, as measured by the gMFI in the PrimeFlow RNA assay. While variation was observed in the frequency of virus positive cells for single, dual and triple infections between cell types, 2a-strains became predominant over time regardless of cell type. Whereas the 2a (PI28) strain outcompeted the 2a (PI12)-strain in BL-3 cells. In BTU cells a small percentage of strain PI12 positive cells did exist on day 30 in dual infection with 2a (PI28)- strain. It is not known if further passage and evaluation would have led to reemergence and predominance of strain PI12. This study clearly demonstrated that the 2a-strains predominated and corroborated the *in vivo* results in which the 2a-strains accounted for over 50% of the live born PI calves (Walz et al., 2018). The PrimeFlow RNA assay also allowed the observation of the dynamics between the 1a and 1b isolates in dual infections. Results from this study suggest limited competition between the 1a and 1b-strains in dual infections in BTu cells as demonstrated by the large proportion of dual positive cells and similar infection rates for each virus. In contrast, by day 30 the 1b-strain had risen to predominate in the BL-3 cells. However, there was a discrepancy between the Prime Flow assay and sequencing results in dual BVDV1 infections in BTu cells. This discrepancy could be explained by the increased number of 1b virus positive cells from day 9 to day 30. Since the gMFI cannot be compared between strains due to differences in fluorescent intensity, the authors cannot confirm the amount of virus present in each respective cell. One possible explanation was there were more viral transcripts for the 1b virus in addition to the increase in the number of 1b virus positive cells from day 9 to day 30 leading to a higher detection of the 1b-strain by sequencing. It is unknown if continued passage of the BTu cells dual infected with the 1a and 1b-strains would have resulted in the 1b strain predominating as observed in the BL-3 cells.

Based on *in vivo* studies it appears that there is viral exclusion between BVDV strains. Only one pestivirus strain can be detected in most animals that had been inoculated with a mixture of two or three pestivirus strains (Brock and Chase, 2000; Makoschey and Janssen, 2011; Peddireddi et al., 2018; Zimmer et al., 2002). While one of those studies demonstrated simultaneous experimental infection with BVDV-1 and BVDV-2 resulted in dual persistent infections as determined by detection of both viruses, both viruses could not be isolated from the same tissue (Brock and Chase, 2000). Numerous studies have described a greater number of animals or tissues to be positive for BVDV-2 strains when used in dual exposure with BVDV-1 strains (Frey et al., 2002; Makoschey and Janssen, 2011; Peddireddi et al., 2018; Walz et al., 2018). It has been hypothesized that there could be differences in level of replication, adaptation, pathogenesis and tissue tropism between BVDV-1 and BVDV-2, during fetal infections (Bielefeldt-Ohmann et al., 2008; Brock and Chase, 2000; Makoschey and Janssen, 2011). Previous studies have described some differences between strains, including a higher degree of viremia, more pronounced lesions and more extensive distribution of viral antigen in calves inoculated with BVDV-2 when compared with calves infected with BVDV-1 (Walz et al., 2001a,b). Further experimentation using a greater number of strains needs to be done to determine if these differences are true of all strains within each species.

This study demonstrated that the PrimeFlow assay is a superior tool for studying coinfections. RT-qPCR and DNA sequencing were unable to distinguish two or more strains in coinfections, where only the strain in a greater proportion was identified and the strain with a low viral load could not be detected. The results from the PrimeFlow RNA assay highlights the issue of the inability of traditional methods to detect mixed infections in contaminated cell cultures. Additionally, another practical application of this novel technique is the evaluation of interactions between strains that can be used in multivalent modified-live virus vaccine, but also as a model for predicting which strains could predominate in the field. The current assay may provide opportunities to evaluate potential vaccine failures or isolates that may be more prone to induce vaccine failure. Finally, these findings may help explain variations observed in the frequency of subgenotypes detected in the field.

## Conflicts of interest

None declared.

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## Appendix A. Supplementary data

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