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INFLUENCE OF TYPE 2 BOVINE VIRAL DIARRHEA VIRUS N^{PRO} ON ENHANCEMENT OF BOVINE RESPIRATORY SYNCYTIAL VIRUS REPLICATION MEDIATED BY ANTAGONISM OF HOST CELL INTERFERON TYPE I RESPONSES

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Bovine viral diarrhea virus (BVDV) is a member of the genus *Pestivirus*, Family *Flaviviridae.* The virus can infect many species of animals of the order *Artiodactyla*. The BVDV genome encodes an auto protease, N^{pro}, that degrades interferon regulatory factor-3 (IRF-3) reducing type I interferon (IFN-I) production from host cells. Bovine respiratory syncytial virus (BRSV) is a member of the genus *Pneumovirus*, Family *Paramyxoviridae*. Concurrent infection with BVDV and BRSV causes more severe respiratory and enteric disease than infection with either virus alone. Our hypothesis was that N^{pro} modulates the innate immune responses to BVDV infection and enhances replication of BVDV or BRSV co-infection. The noncytopathic BVDV2 viruses NY93/c N- N^{pro} 18 EGFP (a mutant with modified N^{pro} fused with enhanced green fluorescent protein), NY93 infectious clone (NY93/c), wild-type NY93-BVDV2 (NY93-wt), and BRSV were evaluated in this study. The objectives of this study were: (1) to characterize the replication kinetics and IFN-I induction in Madin-Darby bovine kidney (MDBK)

cells following infection with each of the BVDV isolates, and (2) to characterize the influence of BVDV-mediated IFN-I antagonism on enhancement of BRSV replication in bovine turbinate (BT) cells. NY93/c N- N^{pro} 18 EGFP replicated 0.4 – 1.6 $TCID_{50}$ logs lower than NY93-wt in MDBK cells. NY93/c N- N^{pro} 18 EGFP-infected MDBK cells synthesized IFN-I significantly higher than NY93/c- and NY93-wt-infected MDBK cells. BT cells co-infected with NY93/c N- N^{pro} 18 EGFP/BRSV or NY93-wt/BRSV were evaluated to determine the effects of co-infection on BRSV replication and IFN-I induction in BT cells. BRSV RNA levels in NY93-wt/BRSV co-infected BT cells were 2.49, 2.79, and 2.89 copy number logs significantly greater than in NY93/c N- N^{pro} 18 EGFP/BRSV co-infected BT cells on days 5, 7, and 9 post-infection, respectively. BVDV RNA levels in NY93/c N- N^{pro} 18 EGFP-infected BT cells were 1.64 – 4.38 copy number logs lower than in NY93-wt-infected BT cells. NY93/c N- N^{pro} 18 EGFP single and co-infected BT cells synthesized IFN-I significantly higher than NY93-wt single and co-infected BT cells. In summary, these findings suggest: (1) NY93/c N- N^{pro} 18 EGFP BVDV2 induced higher levels of IFN-I than BVDV2-wt and may be useful as a safer, replicating BVDV vaccine, and (2) Enhancement of BRSV infection by BVDV co-infection is mediated by antagonism of IFN-I.

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LITERATURE REVIEW

BVDV economic importance

Bovine viral diarrhea virus (BVDV) is a member of the genus *Pestivirus* within the Family *Flaviviridae.* The genus *Pestivirus* also includes classical swine fever virus (CSFV) and border disease virus of sheep (Wengler, 1991). BVDV is commonly associated with cattle, but infection has also been reported in pigs (Liess, 1990; Løken, 1995), deer (Frölich, 1995; Raizman, 2009), other domesticated and exotic ungulates (Løken, 1995), and new world camelids (Belknap, 2000), such as alpacas (Topliff, 2009). BVDV was first identified in New York State from cattle with gastroenteritis, diarrhea, and abortion (Olafson, 1946). BVDV infection is endemic and causes economic loss and considerable animal suffering. In BVDV infected herds, 60-85% of the cattle are antibody positive and 1-2% of the cattle are persistently-infected (PI). PI animals are the common source of the virus and spread the virus by direct contact to other susceptible animals (Houe, 1999). Estimates of economic losses due to BVDV vary depending on the immune status of the animal and the pathogenicity of the infecting virus strains. Annual losses due to BVDV are estimated at $20 per calving due to a low-virulent BVDV strain with an estimated annual incidence of acute infections of 34%. Annual losses are estimated at $75 per calving due to a high-virulent BVDV strain at the same incidence of infection (Houe, 1999). BVDV infection in dairies decreases milk production, reproductive performance and growth while increasing occurrence of other diseases and mortality among calves. At the national level, it is estimated that the losses in dairies due to BVDV infection range between $10 and $40 per calving (Houe, 2003). The mortality

rates associated with infectious disease are significantly higher in feedlot cattle positive for BVDV type I (Booker, 2008).

BVDV types and genome

BVDV exists as two genotypes and two biotypes. Genotypes are designated type I and type II based on sequence differences of the 5' untranslated region (UTR). There are antigenic and pathological differences between type I and type II genotypes (Ridpath, 1994). Monoclonal antibodies against BVDV type II showed no or weak cross-reaction with BVDV type I (Deregt, 1998). In the 5' UTR, two nucleotide substitutions were seen distinguishing between eight isolates of low and high virulence BVDV type II. A cytosine at position 219 and a uracil at position 278 in the low virulence isolates, and the opposite in the high virulence isolates (Topliff, 1998). Strains of BVDV are separated into two biotypes, cytopathic (cp) and noncytopathic (ncp), based on their effects in cell culture (Mendez, 1998). Cp strains of BVDV induce apoptosis of cells *in vitro*, whereas ncp strains do not (Hoff, 1997; Yamane, 2006). Viral proteins of ncp BVDV protect cells from induction of apoptosis by other factors, such as synthetic molecules or other viral infections. Ncp BVDV strains decrease type I interferon (IFN-I) *in vitro* more effectively than cp strains (Schweizer, 2001). Unlike cp BVDV strains, ncp BVDV strains may cause persistent infection (PI) in calves that get infected early in gestation. These PI calves will be immunotolerant and shed virus throughout their lifetime (Bolin, 1990).

BVDV has a single-stranded, positive-sense RNA genome of approximately 12.5 kilobases (kb). It consists of a large open reading frame (ORF) with a UTR on both the 5' and 3' ends (Ridpath, 1995). The BVDV viral proteins from 5' to 3' are in the following order: N^{pro} – C – E^{rns} – E1 – E2 – P7 – NS2 – NS3 – NS4A – NS4B – NS5A – NS5B

(Collett, 1991). The BVDV genome encodes four structural (C, E^{rns}, E1, and E2) and eight non-structural proteins (N^{pro}, P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Donis, 1988; Thiel, 1991; Meyers, 1996; Collett, 1988). The main differences in genomic organization between ncp and cp BVDV are rearrangements in the NS2/NS3 region of the genome. By a variety of mechanisms, such as cellular RNA insertions, cp BVDV arises by RNA recombination of ncp BVDV (Qi, 1992; Tautz, 1994; Ridpath, 1995). Unlike in cp BVDV, the NS2/NS3 region is not cleaved in ncp BVDV (Greiser-Wilke, 1992).

N^{pro} of BVDV and host's interferon

The autoprotease, N^{pro}, is a NS protein that has proteolytic properties and cleaves itself from the capsid protein between Cys_{168} and Ser_{169} of the polyprotein (Stark, 1993). Only *Pestiviruses* within the Family *Flaviviridae* have a NS protein at the N-terminus of their polyproteins. The essential amino acids for the proteolytic activity of N^{pro} are Cys_{69}, His_{49}, and Glu_{22} (Rumenapf, 1998). Attenuated viruses can be made by deleting or mutating the N^{pro} (Tratschin, 1998). The growth kinetics of a BVDV chimeric virus in which the N^{pro} region was replaced with the hepatitis C virus NS3 gene, substituting the N^{pro} enzymatic activity with a serine protease, were similar to the wild-type BVDV. However, the growth kinetics and replication of an N^{pro}-null BVDV virus (entire N^{pro} region deleted) were much lower than the wild-type BVDV, but still viable (Lai, 2000).

Interferons (IFNs), inducible cytokines, are a major component of innate immunity and a bridge between innate and adaptive immunity. They are produced by almost every type of nucleated cell, especially immunological cells including B and T cells. There are three types of IFNs classified as type I, including subtypes α and β, type

II, including subtype γ, and type III, including subtype λ. Type I IFN is the innate immune response to viral infection or dsRNA exposure of cells (Bautista, 2005) and controls some cellular functions such as inducing dendritic cell (DC) differentiation and stimulating proliferation and class switching of B cells (Litinskiy, 2002; Paquette, 2002; Tough, 2004). Type I IFN also stimulates the motility, differentiation, and cross-priming of T cells, enhances the cytotoxicity of natural killer (NK) cells, stimulates the production of inflammatory cytokines or nitric oxide, and affects the capacity of type II IFN to activate phagocytes (Rogge, 1998; Bogdan, 2000; Biron, 2001; Sato, 2001; Le Bon, 2003; Foster, 2004; García-Sastre, 2006). DC, NK, and T helper1 (Th1) cells produce type II IFN. Unlike type I IFN, type II IFN is not directly inducible by viral infection (Munder, 1998). The first characterization of type III IFN (IFN-λ) was in 2002 with similar activity to, but independent of, type I IFN. In several human cell lines and tissues, type III IFN RNA, like type I IFN RNA, was inducible by viral infection (Kotenko, 2003). Type III IFN also has antiviral activity in viral infected animals preventing them from sickness and weight loss (Bartlett, 2005). Type I IFN has control effects on viral infection through its action on cells to assist in the production and activation of constitutively expressed cellular protein kinase R (PKR) (Clemens, 1997; Wang, 2003).

Interferon regulatory factor-3 (IRF-3), an essential antiviral signaling molecule, is a member of the interferon regulatory transcription factor (IRF) family, which is important in IFN regulation and development of the immune system. IRF-3 is expressed in a variety of tissue cells, such as mature DCs. IRF-3 is found in an inactive cytoplasmic form and is posttranslationally-modified following virus infection by protein phosphorylation. This virus-dependent phosphorylation of IRF-3 alters protein

conformation causing the translocation of IRF-3 from the cytoplasm to the nucleus. Phosphorylation also stimulates DNA binding with IRF-3, and increases IRF-3 transcriptional activation and primary activation of IFN-responsive genes to produce type I IFN and establish early innate immunity (Hiscott, 1999; Gabriele, 2007). In both BVDV and CSFV, the amino-terminal cysteine protease, N^{pro}, prevents the production of type I IFN by suppressing levels of IRF-3 to avoid host antiviral responses (Horscroft, 2005). Even though IRF-3 can be relocated from the cytoplasm to the nucleus in BVDV-infected cells, N^{pro} blocks IRF-3 binding to DNA and degrades the protein IRF-3 by proteasomes, but not IRF-3 mRNA. The autoprotease activity of N^{pro} is not required for IFN inhibition (Hilton, 2006; Seago, 2007).

Many studies have evaluated the role of N^{pro} on evasion of the innate immune response of host cells infected with CSFV or BVDV. Host cells produce type I IFN in response to virus infection, and macrophages are important IFN producers (Roberts, 1979). Studies using polyinosinic-polycytidylic acid (poly I:C), a synthetic dsRNA that induces IFN, have shown a novel function of N^{pro} against the cellular innate immune system. Wild-type CSFV protects macrophages and the porcine kidney cells, PK-15, stimulated with poly I:C from poly I:C-induced apoptosis and prevents poly I:C-induced type I IFN production; in contrast, N^{pro}-deleted mutants did not (Ruggli, 2003). Preventing type I IFN production requires the presence of BVDV N^{pro} in cell culture. Infection of DCs, a major link between innate and adaptive immunity, with cp and ncp N^{pro}-deleted CSFV mutants had increased type I IFN production and DC maturation, but the replication of these mutants was decreased (Bauhofer, 2005). In macrophages, monocytes, DCs, and calf testicle cells *in vitro*, type I IFN production was induced in cp

BVDV infection; however, in ncp BVDV infection, type I IFN was suppressed (Adler, 1997; Perler, 2000; Baigent, 2002; Glew, 2003). In pregnant cows experimentally infected with ncp BVDV type II at gestation day 75, type I IFN was increased and virus was cleared in approximately two weeks; however, fetuses were persistently infected (Smirnova, 2008). By antagonism of type I INF, N^{pro} of BVDV plays a role in evasion of the innate immune response. The N-terminal region of N^{pro} is necessary for this antagonism. Abolition of the ability of N^{pro} to suppress production of IFN was achieved by substituting amino acids E22 and H49 (Gil, 2006).

BVDV and innate immunity

The innate immunity is the first line of the host's immune system defying viral infection. Viruses, intracellular pathogens, have developed mechanisms to avoid the innate immunity and allow virus propagation and survival. BVDV double-stranded RNA (dsRNA) is the trigger of the innate immune response. Cp BVDV produces large amounts of dsRNA during viral replication *in vitro*, which stimulates activation of transcriptional factors and increases type I IFN production, while ncp does not (Gil, 2000; Alexopoulou, 2001; Yamane, 2006). Cp and ncp BVDV strains have different effects on the cell pathways of apoptosis and type I IFN production. Ncp BVDV prevents apoptosis to ensure their intracellular survival, while cp BVDV induces apoptosis to insure their spread (Zhang, 1996; Hoff, 1997; Grummer, 1998; Lambot, 1998; Schweizer, 1999). In BVDV infection, the major population of cells undergoing apoptosis is monocytes (Lambot, 1998). NS3 protein of cp BVDV plays a role in induction of apoptosis (St-Louis, 2005). In cells stimulated with poly I:C, a potent inducer of apoptosis, ncp BVDV proteins protect cells from apoptosis (Schweizer, 2001). A study examining the role of

BVDV dsRNA showed that ncp BVDV suppressed apoptosis by inhibiting two dsRNA reactive cellular factors. These cellular factors were dsRNA-dependent protein kinase R (PKR), a molecule that regulates RNA translation in the cell by inactivating the RNA translation factor, eIF2, through phosphorylation, and 2',5'-oligoadenylate synthetase 1 (OAS 1) (Chawla-Sarkar, 2003; Yamane, 2006). In cp BVDV infection *in vitro*, initiation of apoptosis was correlated with inhibition of the anti-apoptotic Bcl-2 protein, induction of expression of caspase-12, and a decrease in intracellular glutathione levels (Jordan, 2002). Ncp BVDV upregulates Bcl-2 anti-apoptosis protein and TNF-α (Bendfeldt, 2003; Yamane, 2005).

BVDV infections

BVDV infections range from subclinical to highly fatal clinical forms, which depend on host immune status, pregnancy status, fetal gestational age, and the influence of concurrent environmental stress factors. BVDV can cause a variety of syndromes including subclinical infections, clinical BVDV with enteric or respiratory disease, hemorrhagic syndrome, reproductive failure, congenital defects, persistent infection, and mucosal disease (Ames, 1986; Brownlie, 1990a; Brownlie, 1990b; Baker, 1995). Early embryonic death, abortion, congenital defects, immunotolerance and birth of seropositive calves are possible consequences of BVDV infection (Kirkbride, 1992). From 15 pregnant heifers experimentally infected with BVDV strains on gestation day 100, six fetuses died *in utero* and five of them were aborted between days 136 and 154 (Done, 1980). Ruminant fetuses (calves and lambs) are very susceptible to transplacental BVDV infection since they are agammaglobulinemic, immunologically immature, and have many immature organ systems with undifferentiated cells. BVDV can cause early

embryonic death, abortion, stillbirth, malformed fetuses, and congenital defects in the central nervous and ocular systems of fetuses. Newborn lambs have low birth weights, are weak, persistently viremic, and immunologically tolerant following exposure of the dam with ncp BVDV (BD-31) during gestation (Osburn, 1991; Hewicker-Trautwein, 1994). Live-born calves show intrauterine retardation and are clinically affected with congenital nervous disease (Done, 1980).

About 70-90% of BVDV infections are subclinical (Ames, 1986). Subclinical-infected dairy cows may exhibit a decrease in milk yield, and more severe respiratory disease in their calves (Moerman, 1994). During acute experimental clinical infection with BVDV type I, calves developed leukopenia, high fever, increased respiratory rates, viremia, and infection of the thymus (Kelling, 2005). Calves acutely infected with BVDV developed mild clinical signs: varying degrees of fever, anorexia, and lymphoid depletion in Peyer's patch. BVDV and its specific antigen were detected with high concentrations in the thymus, Peyer's patch, mesenteric lymph node, and the bone marrow. Platelet counts were significantly reduced and may result in thrombocytopenia during infection (Marshall, 1996).

Acute, PI, and MD BVDV infections result in immunosuppression, which leads to secondary infections by other pathogens (Brodersen, 1998; Gagea, 2006). BVDV potentiates the severity of disease when concurrently infected with other respiratory tract and gastrointestinal pathogens (Kelling, 2002a). One of the most costly diseases in the feedlot cattle industry is bovine respiratory disease complex (BRDC) costing about $14 per animal (Snowder, 2006). BVDV, a significant contributor to BRDC, interacts with several other pathogens, such as bovine respiratory syncytial virus (BRSV). Calves

concurrently infected with BVDV and BRSV had more severe clinical signs of disease and extensive lung lesions, and shed virus in greater concentration for longer duration compared to calves infected with either virus alone. BVDV plays an indirect role by causing host immunosuppression while BRSV acts directly causing bronchopneumonia. BVDV and BRSV possibly act synergistically during co-infection of calves and as a result of synergism, respiratory and digestive tract diseases are enhanced (Brodersen, 1998). BVDV strains are different in causing pneumonia. A ncp BVDV type I (subtype Ib) was the predominant isolate in feedlot cattle with respiratory disease (Fulton, 2002). In enteric infection of neonatal calves, BVDV had direct and indirect roles. Calves with concurrent infection with BVDV and bovine rotavirus (BRV) had more severe enteric infection (villus atrophy and submucosal inflammation) than calves infected with either virus alone (Kelling, 2002a).

Hemorrhagic syndrome (HS) is due to infections with high virulence ncp BVDV type II isolates. The syndrome is characterized by thrombocytopenia, hemorrhage, leukopenia, fever, diarrhea and death (Pellerin, 1994; Ridpath, 1994; Carman, 1998; Kelling, 2002b). Common clinical findings include: diarrhea with blood and mucous, fever, anorexia, depression, dyspnea, and continued bleeding form injection sites. Pathologically, findings may include leukopenia, and thrombocytopenia, but this is not consistently observed in every study (Stoffregen, 2000; Kelling, 2002b). Changes in platelet function were seen in animals infected with BVDV type I and II, and the platelet percentage, especially with type II, was decreased over time up to 12 days post infection (Walz, 2001).

Persistent infection

Ncp BVDV strains can cross the placenta from the pregnant dam to the fetus (Fredriksen, 1999; Harding, 2002). Usually the fetus, infected *in utero* between 42-114 days of gestation, recognizes the viral antigens as a part of its immune system, and becomes persistently-infected and immunotolerant (Malmquist, 1968; McClurkin, 1984) specifically to that BVDV strain, but immunocompetent to other heterologous BVDV strains (Steck, 1980; Bolin, 1985b). Calves with persistent-infection (PI) are important BVDV reservoirs in the environment and shed BVDV lifelong, infecting healthy animals (Houe, 1995). Fetal PI was identified in 0.5% of calves in two dairy herds (Muñoz-Zanzi, 2003) and 0.3% in feedlot herds (Loneragan, 2005). PI animals often show no lesions (Liebler-Tenorio, 2004) and may not be distinguishable from other healthy calves. Some PI animals have increased secondary infections, growth retardation (Barber, 1985; Stokstad, 2002) and increased mortality rates particularly in the first year of life (Duffell, 1985; Houe, 1993). A wide spectrum of pathologic lesions in epithelial and non-epithelial cells may be caused by PI BVDV infections. Histopathological lesions are often rare, but may be seen in the kidneys, hepatic portal triads, lymphatic nodes, and mammary gland. PI calves also may display an abnormal hair coat and have shallow erosions, ulcers, and hemorrhage in the gastrointestinal tract. Oral and abomasal ulcers in cattle with PI have been reported (Bielefeldt-Ohmann, 1995; Shin, 2001). However, the architecture of lymphoid tissues and number of lymphoid cells are normal (Bielefeldt-Ohmann, 1988). BVDV infects many cell types of most organ systems. It can be in neural, epithelial, and lymphoid tissues (Bielefeldt-Ohmann, 1987; Hewicker, 1990). In PI lymphoid cells, BVDV is detected in monocytes and T cells, but not in B cells (Lopez, 1993). In the

central nervous system of PI animals, BVDV was found in the thalamic nuclei, hippocampus, entorhinal cortex, basal nuclei, and piriform cortex (Montgomery, 2007). BVDV has also been detected in the epithelial and non-epithelial cells of the gastrointestinal tract, liver, pancreas, spleen, lymph nodes, lung, kidney, adrenal gland, thymus, mammary glands, macrophages, ovary, uterus, placenta, fetal fluids, and semen (Bielefeldt-Ohmann, 1988; Shin, 2001; Neskanen, 2002; Confer, 2005).

Mucosal disease

Recombination events between ncp and cp BVDV strains can generate new cp strains within PI animals (Bolin, 1995b; Ridpath, 1995; Becher, 1999; Becher, 2001). These new cp BVDV strains superinfect PI animals causing a fatal disease called mucosal disease (MD). Clinical signs of MD include: fever, depression, anorexia, mucous nasal discharge, hemorrhage, weakness, elevated heart and respiratory rates, lesions in the mucosa of the digestive tract, and death (Brownlie, 1984; Bolin, 1985b; Dabak, 2007). Even though MD can be seen in animals of all ages, it is commonly seen between 6 months and two years (Bolin, 1995b).

There are early onset and late onset MDs, depending on the duration between superinfection and the development of MD. If the cp isolate is closely related to the ncp BVDV isolate present in the PI animal, the cp isolate will result in a rapid, early onset MD, which can occur within two weeks post inoculation (Moennig, 1990). On the other hand, if there is less antigenic homology between ncp and cp isolates, a recombination event between them must occur to develop a late onset MD, which can occur within months post inoculation (Fritzeneier, 1995; Fritzemeier, 1997). There are minor pathological differences between the two forms of MD. Vascular lesions were observed

in late onset MD, but were absent in early onset MD. The histological lesions are similar in both, but are different in distribution. Severe depletion of Peyer's patches was observed in both courses, but there was a complete loss of lymphoid architecture in late onset MD only (Liebler-Tenorio, 2000).

BVDV transmission and control

The main source of BVDV infection is PI animals that shed virus in the environment and transmit it to healthy animals by direct contact; however, acutely infected animals secrete and transmit virus, but only for a short time. Also, other species of animals infected with BVDV can transmit virus. Transmission by indirect contact may happen through the use of contaminated equipment, such as needles and gloves (Houe, 1999; Fulton, 2005). BVDV transmission can also occur through insemination with BVDV infected semen that has normal quality. Serving seronegative dams with semen from PI bulls results in poor rates of conception and may result in PI calves (Paton, 1990; Kirkland, 1994; Niskanen, 2002; Givens, 2003). Furthermore, exchange of embryos, gametes, semen, and somatic cells provide an unnatural way to transmit BVDV between herds of cattle over the world (Gard, 2007).

Identification and elimination of PI animals and vaccination are important to prevent BVDV infections (Moennig, 2005). Quarantine of new animals and testing them for BVDV PI status before accepting them is very important to maintain a closed herd (Kelling, 2000; Brock, 2004). Modified live and inactivated vaccines are widely used and because of the antigenic variability, both contain type I and type II BVDV strains (Bolin, 1995a; Beer, 1997). The timing of BVDV vaccination depends on factors that include: immune response, crossreactivity, fetal protection, immunosuppression, duration of

immunity, reversion to virulence, effect of maternal antibody on immune responses and purity. There is no single BVDV vaccine that provides complete fetal protection (Kelling, 2004).

There are several methods to diagnose BVDV infections or to confirm the vaccine efficacy in live animals or to identify contamination in biological products. These tests include: reverse transcription-polymerase chain reaction (RT-PCR), antigen detection by fluorescent antibody (FA) testing, enzyme-linked immunosorbent assay (ELISA), antigen capture ELISA (ACE), immunohistochemistry (IHC), and virus isolation (VI). Virus isolation is considered the best for BVDV diagnosis (Saliki, 2004; Sandvik, 2005). Real-time PCR and reverse transcriptase PCR (RT-PCR) are used to detect BVDV and for genotyping (Ridpath, 1998). IHC and ACE rarely miss a PI animal. They detected 100% of PI calves. Since the tests sometimes were positive for acute infections, using virus isolation or RT-PCR is recommended 30 days after the initial test to confirm PI infections (Cornish, 2005). A study evaluated the diagnostic proficiency of methods for detecting BVDV in infected cattle using comparisons among tests and laboratories, the test that provided the greatest consistency in detecting positive animals is ACE. It also has a very good agreement among diagnostic laboratories (Edmondson, 2007).

RT-PCR and Q-RT-PCR

RT-PCR is a very sensitive method for virus identification and differentiation. Quantitative RT-PCR (Q-RT-PCR) determines the total amount of viral RNA present in a sample (Menzo, 1992). RT-PCR is a quick diagnostic method compared to other methods such as virus isolation, plaque assay, or viral titration. The first amplification of extracted cp and ncp BVDV RNA was in 1990 using BVDV infected cell cultures and clinical

samples from PI and acutely-infected calves. The amplified RNA was visualized using agarose gel electrophoresis and staining with ethidium bromide or hybridization with biotinylated probes. RT-PCR is specific and sensitive. RT-PCR can be used with different primer sets to distinguish between strains of viruses. It is possible to determine BVDV genotype, I or II, in a sample using RT-PCR (Schroeder, 1990; Belák, 1991; Hertig, 1991; Schmitt, 1994; Tajima, 1995). Nevertheless, gel-based RT-PCR results can be compromised during nucleic acid amplification by sample contamination.

A single tube, fluorogenic probe-based, real-time quantitative RT-PCR (Q-RT-PCR), TaqMan RT-PCR, was developed to detect RNA of classical swine fever virus (McGoldrick, 1999; Ophuis, 2006). Q-RT-PCR is a rapid, economical, high volume, sensitive, and specific procedure to detect, quantify, and classify many viruses in samples. Q-RT-PCR has been used to quantify and classify BVDV in serum and tissue homogenate (Bhudevi, 2001; Mahlum, 2002). Using BioRad's iCycler iQ, real-time Q-RT-PCR was used to detect and quantify viral mRNA of bovine respiratory syncytial virus (BRSV) in cell lysate harvested at different time points post-infection. Real-time Q-RT-PCR was specific, rapid, and efficient, and eliminating the post-PCR processing steps compared to quantitative competitive RT-PCR (QC-RT-PCR) (Achenbach, 2004). QC-RT-PCR is an appropriate method for diagnosis of diseases and evaluation of the efficiency of vaccines (Boxus, 2005).

IFN assays

Reporter gene assays have been developed to measure type I IFN production. The Mx reporter gene assay uses a plasmid containing a human MxA promoter associated with chloramphenical acetyltransferase (CAT) cDNA. This plasmid is transfected into Madin-Darby Bovine Kidney (MDBK) cells. The IFN in test samples will stimulate the Mx promoter which results in CAT synthesis. CAT is measured using a commercial ELISA kit which reflects the amount of IFN in the test sample (Fray, 2001). In the luciferase reporter assay, the reporter cell line, NCL1-ISRE-Luc-Hygro, are used. These cells are bovine uterus cells transfected with a plasmid containing an IFN stimulated response element (ISRE) promoter with luciferase reporter gene. Test samples are added to these cells and IFN-I is quantified using a luciferase assay reagent (Gil, 2006). In addition to reporter gene assays, real-time reverse transcriptase polymerase chain reaction (Real-time RT-PCR) has been used to measure the transcription of IFN-related RNA (Yamane, 2008).

BRSV

Bovine respiratory syncytial virus (BRSV) is a member of the genus *Pneumovirus* within the Family *Paramyxoviridae* (Murphy, 1995). BRSV is closely related to human respiratory syncytial virus (HRSV). Respiratory syncytial virus (RSV) has a single-strand, negative-sense RNA genome of 15,222 nucleotides encoding 11 proteins (Huang, 1982). All of the BRSV mRNAs except one, BRSV polymerase, correspond to HRSV mRNA. Unlike F, N, M, and P proteins, only G glycoprotein of BRSV showed major antigenic differences from the G glycoprotein of HRSV (Lerch, 1989). The determinants of BRSV host range are the nonstructural proteins, NS1 and NS2. These proteins together

inhibit the phosphorylation and transcriptional activity of IRF-3 resisting type I IFN production (Bossert, 2002; Bossert, 2003). In 1955, RSV was first reported in chimpanzees as a respiratory disease (Blount, 1956). Therefore, it was called chimpanzee coryza agent, and since it forms syncytia, became called RSV (Chanock, 1957). In cattle, BRSV was described as a pathogen of the respiratory system (Paccaud, 1970). BRSV infection commonly occurs during the first year of life in calves, but reinfection can occur at any age (Van der Poel, 1993). In small ruminants, sheep and goats, there are related RSV isolates causing respiratory tract diseases (Mallipeddi, 1993; Oberst, 1993; Yeşilbağ, 2009).

Clinical symptoms of severe natural BRSV infection include: fever, cough, and increased respiratory rates. In lungs, the lesions include bronchitis, bronchiolitis, fibrosis, emphysema, and severe edema (Bryson, 1983; Kimman, 1989). In experimental infection with BRSV, calves had fever and diarrhea and developed lobular suppurative and necrotic bronchointerstitial pneumonia and diffuse cilia loss with mild necrosuppurative inflammatory changes (Brodersen 1998). Colostrum-fed 17- to 24-day-old calves experimentally infected with BRSV developed severe chronic bronchiolitis and bronchiolitis obliterans. Secretions filled the bronchiolar lumina and alteration of the ciliogenesis was observed with partial loss of cilia (Philippou, 2000). In calves with BRDC, concurrent infection with BVDV and BRSV causes more severe respiratory and enteric disease than infection with either virus alone (Brodersen, 1998).

THESIS

INFLUENCE OF TYPE 2 BOVINE VIRAL DIARRHEA VIRUS N^{PRO} ON ENHANCEMENT OF BOVINE RESPIRATORY SYNCYTIAL VIRUS REPLICATION MEDIATED BY ANTAGONISM OF HOST CELL INTERFERON TYPE I RESPONSES

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ABSTRACT

Bovine viral diarrhea virus (BVDV) is a member of the genus *Pestivirus*, Family *Flaviviridae.* The virus can infect many species of animals of the order *Artiodactyla*. The BVDV genome encodes an auto protease, N^{pro}, that degrades interferon regulatory factor-3 (IRF-3) reducing type I interferon (IFN-I) production from host cells. Bovine respiratory syncytial virus (BRSV) is a member of the genus *Pneumovirus*, Family *Paramyxoviridae*. Concurrent infection with BVDV and BRSV causes more severe respiratory and enteric disease than infection with either virus alone. Our hypothesis was that N^{pro} modulates the innate immune responses to BVDV infection and enhances replication of BVDV or BRSV co-infection. The noncytopathic BVDV2 viruses NY93/c N- N^{pro} 18 EGFP (a mutant with modified N^{pro} fused with enhanced green fluorescent protein), NY93 infectious clone (NY93/c), wild-type NY93-BVDV2 (NY93-wt), and BRSV were evaluated in this study. The objectives of this study were: (1) to characterize the replication kinetics and IFN-I induction in Madin-Darby bovine kidney (MDBK) cells following infection with each of the BVDV isolates, and (2) to characterize the influence of BVDV-mediated IFN-I antagonism on enhancement of BRSV replication in bovine turbinate (BT) cells. NY93/c N- N^{pro} 18 EGFP replicated 0.4 – 1.6 $TCID_{50}$ logs lower than NY93-wt in MDBK cells. NY93/c N- N^{pro} 18 EGFP-infected MDBK cells synthesized IFN-I significantly higher than NY93/c- and NY93-wt-infected MDBK cells. BT cells co-infected with NY93/c N- N^{pro} 18 EGFP/BRSV or NY93-wt/BRSV were evaluated to determine the effects of co-infection on BRSV replication and IFN-I induction in BT cells. BRSV RNA levels in NY93-wt/BRSV co-infected BT cells were 2.49, 2.79, and 2.89 copy number logs significantly greater than in NY93/c N- N^{pro} 18

EGFP/BRSV co-infected BT cells on days 5, 7, and 9 post-infection, respectively. BVDV RNA levels in NY93/c N- N^{pro} 18 EGFP-infected BT cells were 1.64 – 4.38 copy number logs lower than in NY93-wt-infected BT cells. NY93/c N- N^{pro} 18 EGFP single and co-infected BT cells synthesized IFN-I significantly higher than NY93-wt single and co-infected BT cells. In summary, these findings suggest: (1) NY93/c N- N^{pro} 18 EGFP BVDV2 induced higher levels of IFN-I than BVDV2-wt and may be useful as a safer, replicating BVDV vaccine, and (2) Enhancement of BRSV infection by BVDV co-infection is mediated by antagonism of IFN-I.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a member of the genus *Pestivirus* within the Family *Flaviviridae* (Wengler, 1991). Bovine respiratory syncytial virus (BRSV) is a member of the genus *Pneumovirus* within the Family *Paramyxoviridae* (Murphy, 1995). BVDV is commonly associated with cattle, but also infects some other members of the order *Artiodactyla* (Løken, 1995; Raizman, 2009; Topliff, 2009). BVDV infection is endemic and causes significant economic loss. In BVDV infected herds, 60-85% of the cattle are antibody positive and 1-2% of the cattle are persistently-infected (PI). PI animals are the common source of the virus and spread the virus by direct contact with healthy animals. BVDV infection in dairies decreases milk production, reproductive performance and growth while increasing occurrence of other diseases and mortality among calves (Houe, 1999; Houe, 2003). The mortality rates associated with infectious disease are significantly higher in feedlot cattle positive for BVDV type I (Booker, 2008). BVDV potentiates the severity of disease when concurrently infected with other respiratory tract and gastrointestinal pathogens, such as BRSV (Kelling, 2002). BRSV infection commonly occurs during the first year of life in calves, but reinfection can occur at any age (Van der Poel, 1993). In calves, concurrent infection with BVDV and BRSV causes more severe respiratory and enteric disease than infection with either virus alone (Brodersen, 1998).

BVDV exists in two genotypes, type I and type II, based on sequence differences of the 5' untranslated region (UTR) (Ridpath, 1994). BVDV is also separated into two biotypes, cytopathic (cp) and noncytopathic (ncp), based on its effects in cell culture (Mendez, 1998). BVDV is an enveloped virus and has a single-stranded, positive-sense

RNA genome of approximately 12.5 kb. It consists of a large open reading frame (ORF) with a UTR on both the 5' and 3' ends (Ridpath, 1995; Hulst, 1997). The BVDV viral proteins from 5' to 3' are in the following order: N^{pro}, C, E^{rns}, E1, E2, P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Collett, 1991).

Interferons (IFNs), inducible cytokines, are a major component of innate immunity and a bridge between innate and adaptive immunity. Type I IFN is the innate immune response to viral infection or dsRNA exposure of cells (Bautista, 2005) and activates cellular factors to degrade viral RNA and shut down viral mRNA synthesis (Castelli, 1997). Type I IFN controls some cellular functions such as inducing dendritic cell (DC) differentiation and stimulating proliferation and class switching of B cells (Litinskiy, 2002; Paquette, 2002; Tough, 2004). Type I IFN also stimulates the motility, differentiation, and cross-priming of T cells, enhances the cytotoxicity of natural killer (NK) cells, stimulates the production of inflammatory cytokines or nitric oxide, and affects the capacity of type II IFN to activate phagocytes (Rogge, 1998; Bogdan, 2000; Biron, 2001; Sato, 2001; Le Bon, 2003; Foster, 2004; García-Sastre, 2006).

The autoprotease, N^{pro}, is a nonstructural protein that has an important role on evasion of the innate immune response. Classical swine fever virus (CSFV) with a functional N^{pro} prevents type I IFN production from infected cells while N^{pro}-deleted mutants did not (Ruggli, 2003). BVDV N^{pro} prevents the production of type I IFN by suppressing levels of interferon regulatory factor-3 (IRF-3) to avoid host antiviral responses (Horscroft, 2005). Abolition of the ability of BVDV N^{pro} to suppress production of IFN was achieved by substituting amino acids E22 and H49 (Gil, 2006).

BVDV with a modified N^{pro} created by inserting the enhanced green fluorescent protein (EGFP) between amino acids 18 and 19 of the N^{pro} protein, BVDV-wt, and BRSV were used in this study to characterize the effects of BVDV N^{pro} on type I IFN production from infected Madin-Darby bovine kidney (MDBK) cells. The effects of the BVDV N^{pro} on BRSV titers and RNA synthesis, and type I IFN production in BRSV co-infected bovine turbinate (BT) cells were also compared.

MATERIALS AND METHODS

Cells and viruses

Bovine turbinate (BT, National Veterinary Services Laboratory, USDA, Ames, IA) and Madin-Darby bovine kidney (MDBK, CCL-22, American Type Culture Collection, Manassas, VA) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% equine serum (Hyclone, Logan, UT). NCL1-ISRE-Luc-Hygro cells, a modified bovine uterine cell line constitutively expressing an IFN response element gene coupled with firefly luciferase, were maintained in DMEM supplemented with 10% equine serum and 300 µg/ml hygromycin (Cellgro, Manassas, VA).

Non-cytopathic BVDV type 2 isolates consisted of NY93 (Animal, Plant, and Health Inspection Service, Center for Veterinary Biologics, Ames, IA), NY93/c (Meyer, 2002), and NY93/c N-N^{pro} 18 EGFP. NY93/c N-N^{pro} 18 EGFP (Figure 1) was constructed by mutating NY93/c between N^{pro} codons 18 and 19 creating an NaeI restriction site. The EGFP coding region was then PCR amplified from pEGFP-N1 (Clontech, Mountain View, CA) and ligated into the NaeI site between codons 18 and 19.

The bovine respiratory syncytial virus (BRSV) consisted of the field isolate 236-652 (Brodersen, 1998).

Viral stocks

MDBK cell monolayers at 90% confluency were individually infected with each BVDV isolate at a multiplicity of infection (m.o.i.) of 1 in 162 cm^2 tissue culture flasks and incubated at 37°C in a humidified incubator with 5% CO_2 for four days. Following a single freeze-thaw cycle, 1 or 2 ml aliquots of mock- and viral-infected MDBK cell lysates were stored at – 80ºC.

BT cell monolayers at 90% confluency were infected with BRSV at an m.o.i. of 1 in 162 cm^2 tissue culture flasks and incubated at 37°C in a humidified incubator with 5% CO_2 for eight to ten days until cytopathic effects (CPE) were observed. Following a single freeze-thaw cycle, 2 ml aliquots of viral-infected BT cell lysates were stored at – 80ºC.

dsRNA stock

Polyriboinosinic polyribocytidylic acid (poly I:C) (Amersham Biosciences, Piscataway, NJ) was reconstituted in phosphate buffered saline (PBS) to a stock concentration of 2 mg/ml, passed through a 21 gauge needle to shear the RNA, and stored in aliquots at – 80°C.

BVDV growth kinetics

MDBK cells were seeded onto 6-well plates and incubated at 37°C in a humidified incubator with 5% CO_2 until 90% confluent. Cells were then infected with BVDV isolates, NY93-wt, NY93/c, or NY93/c N-N^{pro} 18 EGFP, at an m.o.i. of 0.01 and incubated at 37°C for 1.5 hours with rocking. Following adsorption, cells were washed

with DMEM and incubated in fresh media supplemented with 5% equine serum for BT cells. Cells were monitored by microscopic examination and frozen at – 80°C 0, 1.5, 3, 6, 9, 12, 18, 24, 48, 72, or 96 hours post-infection. Cell lysates were thawed on ice and virus titers determined at each time-point (Brodersen and Kelling, 1999).

BRSV growth kinetics in single and co-infected cells

BT cells were seeded onto 6-well plates and incubated at 37°C in a humidified incubator with 5% CO_2 until 90% confluent. Cells were then infected with BRSV and/or BVDV isolates, NY93-wt or NY93/c N-N^{pro} 18 EGFP, at an m.o.i. of 0.6 and incubated at 37°C for 1.5 hours with rocking. Following adsorption, cells were washed with DMEM and incubated in fresh media supplemented with 5% equine serum. Cells were monitored by microscopic examination and frozen at – 80°C 1.5, 6, 12, and 24 hours, and 2, 3, 5, 7, and 9 days post-infection. Cell lysates were thawed on ice, virus titers determined and RNA extraction performed at each time-point.

Virus titration

Serial ten-fold (10^{-1} – 10^{-8}) dilutions of virus in DMEM were assayed. An aliquot (50 µl) of each dilution was added to each of 8 wells/dilution of a 96-well tissue culture plate. To each well, a 100µl suspension of MDBK cells (2 x 10^4 cells/well) for BVDV or BT cells (1 x 10^4 cells/well) for BRSV, in DMEM-supplemented with 3% horse serum, 75µg of gentamicin/ml, and 0.375µg of amphotericin B/ml. After incubating for 4 days for BVDV or 7 days for BRSV at 37°C in a humidified incubator with 5% CO_2, cells were fixed in 20% acetone (v/v) in PBS for 15 minutes at room temperature and plates allowed to dry overnight. An immunoperoxidase staining protocol (Brodersen, 1998) was performed using the monoclonal antibody 348, specific for BVDV gp53 (VMRD,

Inc. Pullman, WA), or 8G12, specific for BRSV F protein (Klucas, 1988), as the primary antibody and biotinylated horse anti-mouse immunoglobulin (Vector Laboratories, Burlingame, CA) as the secondary antibody. Antibody binding was detected using streptavidin horseradish peroxidase (HRP) conjugate (Zymed, San Francisco, CA) and 3-amino-9-ethyl-carbazole (AEC) to complete the staining procedure.

Virus preparation for IFN assay

Virus preparation for the IFN assay has been previously described (Gil, 2006). Briefly, MDBK cells were inoculated with BVDV isolates: NY93-wt, NY93/c, or NY93/c N-N^{pro} 18 EGFP, at an m.o.i. of 1 and incubated at 37°C for 4 days, then frozen at – 80°C. Flasks were thawed, cell lysates harvested and centrifuged at 2,000 x g for 30 minutes at 4°C to clarify the supernatant. The supernatant was then transferred to ultracentrifuge tubes and centrifuged at 100,000 x g for 2 hours at 4°C. The resulting viral pellet was re-suspended in 500 µL DMEM and stored in aliquots at – 80°C.

IFN reporter gene assay

MDBK cells were seeded onto 6-well plates at a cell density of 3 x 10^5 and grown to 90% confluency. Cells were then infected with pelleted virus isolates at an m.o.i. of 0.5 and incubated at 37°C for 24 hours, then frozen at – 80°C. The plates were thawed and the cell lysate supernatant clarified by centrifugation at 2000 x g for 30 minutes at 4°C. The pH of the clarified supernatant was adjusted to pH 2 with 2 M HCl. After incubation at 4°C for 24 hours, the pH of the supernatant was adjusted to pH 7 using 2 M NaOH. Test samples (0.5 ml) were added to 12 well plates of NCL1-ISRE-Luc-Hygro cells (Gil, 2006) prepared 12 hours earlier by adding 1.5 x 10^5 cells/well and incubating at 37ºC. After addition of the test sample, the NCL1-ISRE-Luc-Hygro cells

were incubated for 8 hours, followed by cell lysis in 100 µl of passive lysis buffer (Promega, Madison, WI) (Gil, 2006). The luciferase assay was performed using the firefly luciferase assay system (Promega) according to the manufacturer's instructions, with sample luminescence measured by a FLUOStar luminometer (BMG Labtech, Offenburg, Germany).

IFN inhibition assay

MDBK cells were seeded onto 6-well plates at a cell density of 3 x 10^5 and grown to 90% confluency. Cells were then infected with BVDV isolates: NY93-wt, NY93/c, or NY93/c N-N^{pro} 18 EGFP, at an m.o.i. of 1.2 and incubated for 48 hours at 37°C, at which time poly I:C was added to the cells at a concentration of 50 µg/ml (Baigent, 2002; Gil, 2006). Following incubation for 20 hours at 37°C, cells were harvested by a freeze-thaw cycle at – 80°C and pH adjusted to pH 2 for 24 hours at 4°C to inactivate virus. The pH of the cell lysates was then adjusted to pH 7 and added to reporter cell plates containing NCL1-ISRE-Luc-Hygro cells and assayed as described previously (Gil, 2006).

RNA extraction using TRIZOL® LS

Total RNA was extracted from cell lysates of BVDV-infected cell cultures using TRIZOL® LS Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Extracted RNA was stored in 75% ethanol at – 80°C until used. Prior to use, RNA was pelleted, dried, and resuspended in 10µl diethylpyrocarbonate-treated (DEPC-treated) water and used immediately.

RT-PCR Primers

Primers for BVDV and EGFP amplification were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). The primers for BVDV amplification were: forward

primer: 5’ CAT GCC CAT AGT AGG AC 3’ and reverse primer: 5’ CCA TGT GCC ATG TAC AG 3’ (Ridpath, 1998).

The primers for EGFP amplification were: forward primer: 5’ GTG AGC AAG GGC GAG GAG CTG 3’ and reverse primer: 5’ CTT GTA CAG CTC GTC CAT GCC GAG AG 3’.

Reverse transcription polymerase chain reaction (RT-PCR) assay

The RT-PCR assay was used to verify the presence of BVDV or EGFP in viral stocks. Total RNA was extracted from BVDV infected cell lysates using Trizol® LS reagent (Invitrogen, Carlsbad, CA). Extracted RNA was stored in 75% ethanol at – 80ºC until used. Prior to use, RNA was pelleted, dried, and resuspended in 10 µl DEPC-treated water and used immediately for RT-PCR. RT-PCR and primers were used to identify BVDV as previously described (Ridpath, 1998). Briefly, RNA was reverse-transcribed to generate complementary DNA (cDNA) using Superscript® III reverse transcriptase (Invitrogen, Carlsbad, CA) and BVDV first-round downstream primer (Ridpath, 1998). BVDV cDNA was amplified using Taq DNA polymerase (Invitrogen, Carlsbad, CA), with first-round upstream and downstream primers. The BVDV specific primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). The reaction mixture was denatured at 94ºC for 10 seconds, annealed at 50ºC (or 55ºC for EGFP) for 15 seconds and extended at 72ºC for 30 seconds for a total of 30 cycles, followed by an additional extension of 10 minutes at 72ºC. Amplified PCR products were visualized by ethidium bromide stained agarose gel electrophresis.

RNA extraction using QIAamp® Viral RNA Mini kit (spin protocol)

BVDV or BRSV total RNA was extracted from 140 µl BT cell lysate infected with BVDV-wt or NY93/c N-N^{pro} 18 EGFP, with and without BRSV, and BRSV alone from each time point using the QIAamp® Viral RNA Mini kit (spin protocol) (Qiagen, Valencia, CA) according to the manufacturer's directions. Extracted RNA was stored in AVE buffer at – 80°C until used.

Real-time RT-PCR Primers and Probe

Primers and probes for BVDV and BRSV amplification were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). The primers and probe for BVDV amplification were: forward primer: 5'-GGGNAGTCGTCARTGGTTCG-3'; reverse primer: 5'-GTGCCATGTACAGCAGAGWTTTT-3'; and probe: 5'-6-FAM-CCAYGTGGACGAGGGCAYGC-TAMRA-3'. Primers and probe were based on sequences of the 5' UTR (Mahlum, 2002).

The primers and probe for BRSV amplification were: forward primer: 5'-GCA-ATG-CTG-CAG-GAC-TAG-GTA-TAA-T-3'; reverse primer: 5'-ACA-CTG-TAA-TTG-ATG-ACC-CCA-TTC-T-3'; and probe: 5'-/5HEX/-ACC-AAG-ACT-TGT-ATG-ATG-CTG-CCA-AAG-CA-/31ABkFQ/-3'. Primers and probe were based on conserved regions of the published sequences of the BRSV N gene (Boxus, 2005).

Real time Q-RT-PCR

Real-time Q-RT-PCR was performed in single wells of a 96-well plate (Bio-Rad, Hercules, CA, USA) in a 25 µl reaction volume using components of a commercial RT-PCR kit (QuantiTect Probe RT-PCR kit, Qiagen, Valencia, CA, USA).

For BVDV, the 25µl reaction mixture contained: 12.5µl QuantiTect Probe RT-PCR Mix (2X), 1µl of 12.5µM forward primer, 1µl of 12.5µM reverse primer, 1µl of 7.5µM fluorogenic probe, 4.25µl of RNase-Free Water, 0.25µl of QuantiTect RT Mix, and 5µl of BVDV RNA sample. The RT-PCR thermocycling program consisted of 50°C for 30 minutes, 95°C for 15 minutes, followed by 40 cycles of 95° C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds.

For BRSV, the 25µl reaction mixture contained: 12.5µl QuantiTect Probe RT-PCR Mix (2X), 1µl of 10µM forward primer, 1µl of 10µM reverse primer, 1µl of 5µM fluorogenic probe, 4.25µl of RNase-Free Water, 0.25µl of QuantiTect RT Mix, and 5µl of BRSV RNA sample. The RT-PCR thermocycling program consisted of 50°C for 30 minutes, 95°C for 15 minutes, followed by 45 cycles of 94°C for 15 seconds and 59°C for 60 seconds . Both reverse transcription and PCR were carried out in the same well of a 96-well plate using a commercial thermocycler.

Fluorescence was measured following each cycle and displayed graphically. The software determined a cycle threshold (Ct) value, which identified the first cycle at which the fluorescence was detected above the baseline for that sample or standard.

The standard curve, Ct value vs. starting RNA amounts from serial viral dilution was used to determine the initial starting quantity of unknown BVDV or BRSV RNA from each time point based on the Ct values for the known BVDV or BRSV standards.

Statistical analyses

Statistical analyses were carried out with the SAS statistical software program (Cary, NC, USA) using the student T test and analysis of variance (ANOVA). The level of significance was set at 0.05.

RESULTS

Viral titration of BVDV isolates

The growth kinetics of the parental, clone, and mutated BVDV NY93 virus isolates were evaluated from cell MDBK lysates harvested at 0, 1.5, 3, 6, 9, 12, 18, 24, 48, 72, and 96 hours post-infection with each virus (Figure 2). Growth kinetics of NY93-wt and NY93/c showed similar viral replication ($p>0.8199$) between 12 and 96 hours post-infection. Between 18 and 96 hours post-infection, NY93/c N-N^{pro} 18 EGFP replicated 0.4 – 1.6 $TCID_{50}$ logs lower than NY93-wt ($p>0.2067$) and 0.7 – 1.1 $TCID_{50}$ logs lower than NY93/c ($p>0.1879$). NY93-wt and NY93/c virus isolates were not detected before 9 hours post-infection, and NY93/c N-N^{pro} 18 EGFP virus was not detected before 12 hours post-infection.

IFN response of MDBK cells to BVDV infections

The IFN responses of MDBK cells to BVDV NY93-wt, NY93/c, and NY93/c N-N^{pro} 18 EGFP were evaluated using the NCL1-ISRE-Luc-Hygro reporter cell line. Cells infected with NY93-wt or NY93/c synthesized equivalent levels of IFN compared to negative control cells; however, cells infected with the NY93/c N-N^{pro} 18 EGFP isolate synthesized significantly ($p<0.0001$) greater levels of IFN compared to negative control cells, NY93-wt and NY93/c-infected cells. The levels of IFN synthesized by cells infected with NY93/c N-N^{pro} 18 EGFP were 14, 14.3, and 17.5 times greater than the negative cells, NY93-wt- and NY93/c-infected cells, respectively (Figure 3). MDBK cells exposed to poly I:C synthesized significantly greater levels of IFN compared to viral infected or negative control cells.

MDBK cells infected with NY93-wt or NY93/c and stimulated 24 hours post-infection with poly I:C synthesized equivalent low levels of IFN. Cells infected with NY93/c N-N^{pro} 18 EGFP synthesized significantly ($p=0.0077$) greater levels of IFN, 2.6 and 3.2 times greater than cells infected with NY93/c and NY93-wt isolates, respectively (Figure 4). Positive and negative control cells synthesized significantly ($p<0.0001$) greater levels of IFN compared to viral infected cells. MDBK cells exposed to poly I:C twice (positive control) synthesized significantly ($p<0.0001$) greater levels of IFN compared to viral infected or negative control stimulated once with poly I:C.

Viral titration in BVDV infected BT cells

The parental (BVDV-wt) and mutated BVDV NY93 (NY93/c N-N^{pro} 18 EGFP) virus isolates replicated to significantly different levels in BT cells. NY93/c N-N^{pro} 18 EGFP replicated 1.65, 2.6, and 4.3 $TCID_{50}$ logs lower than BVDV-wt at 12 hours, 3 days, and 9 days post-infection, respectively ($p=0.0343$) (Figure 5). A similar observation was seen in the quantity of BVDV RNA in cell lysates. Cells infected with NY93/c N-N^{pro} 18 EGFP had 1.48 to 4.26 logs BVDV RNA copy number lower than cells infected with BVDV-wt between 1.5 hours and 9 days post-infection ($p<0.0001$) (Figure 6).

Viral titration in co-infected BT cells

BT cells infected with BVDV NY93-wt alone, NY93-wt/BRSV, NY93/c N-N^{pro} 18 EGFP alone, NY93/c N-N^{pro} 18 EGFP/BRSV, or BRSV alone were assayed for viral infectivity at 1.5 hours, 12 hours, 3 days, and 9 days post-infection for BVDV and at 1.5, 6, 12, and 24 hours, and 2, 3, 5, 7, and 9 days post-infection for BRSV. Growth kinetics of NY93/c N-N^{pro} 18 EGFP with and without BRSV were similar ($p=0.8607$). Growth kinetics of BVDV-wt with and without BRSV were also similar ($p=0.6912$) (Figure 5).

BRSV replicated similarly in cells infected with BRSV alone, BRSV/NY93/c N-N^{pro} 18 EGFP, or BRSV/BVDV-wt at all time-points except day 5, where BRSV titer was significantly (p=0.0228) greater in cells infected with BRSV/BVDV-wt compared to other infection (Figure 8).

Viral RNA levels in co-infected BT cells

BT cell lysates were evaluated for viral RNA levels. RNA levels of NY93/c N-N^{pro} 18 EGFP with and without BRSV were similar (p=0.1594). RNA levels of BVDV-wt with and without BRSV were also similar (p=0.2406) (Figure 6). BRSV RNA levels in cells infected with BRSV or BRSV/NY93/c N-N^{pro} 18 EGFP were similar at all time-points (p=0.9243); however, BRSV RNA levels in cells co-infected with BRSV/BVDV-wt were 2.49, 2.79, and 2.89 copy number logs significantly (p=0.0008) greater than in cells infected with BRSV alone or BRSV/NY93/c N-N^{pro} 18 EGFP at time-points 5, 7, 9 days post-infection, respectively, and were similar at earlier time-points, 1.5 hours to 3 days post-infection (Figure 9).

Viral titration and RNA levels in co-infected BT cells

Viral titrations and RNA levels of each infection were compared. Viral titrations and RNA levels of BVDV in cells infected with BVDV-wt with or without BRSV were comparable at p=0.05. Also, there was no significant difference in viral titers and RNA levels of BVDV in cells infected with NY93/c N-N^{pro} 18 EGFP with or without BRSV (Figure 7). BRSV RNA levels in cells infected with BRSV, BRSV/BVDV-wt, or BRSV/NY93/c N-N^{pro} 18 EGFP were significantly greater than BRSV titers in the same infected cells, but they showed the same pattern of values (Figure 10). At time-points

1.5 – 24 hours, BRSV virus infectivity was not detectable, while BRSV RNA was detectable at all time-points.

Real-time Q-RT-PCR

BT cell lysates were evaluated for BVDV and BRSV RNA. Amplification plots and standard curves were generated for each real-time Q-RT-PCR (Figures 12 to 15). The standard curve of BVDV-wt had a slope of – 51.444 and a correlation coefficient (R^2) of 0.997. The standard curve of NY93/c N-N^{pro} 18 EGFP had a slope of – 23.106 and R^2 of 0.986. The standard curve of BRSV had a slope of – 16.073 and R^2 of 0.954. It also had a slope of – 15.770 and R^2 of 0.950.

IFN response of BT cells to BVDV, BRSV, or to BVDV/BRSV co-infections

The IFN response of BT cells to NY93-wt (BVDV-wt), NY93/c N-N^{pro} 18 EGFP, and BRSV alone and in co-infections were evaluated using the NCL1-ISRE-Luc-Hygro reporter cell line (Figure 11). Cells infected with NY93/c N-N^{pro} 18 EGFP had IFN levels 2.55 times significantly ($p=0.0013$) greater than cells infected with BVDV-wt, and 1.35 times greater than negative control cells. Negative control cells synthesized twice the amount of IFN compared to cells infected with BVDV-wt isolate. BT cells exposed to poly I:C synthesized significantly ($p<0.0001$) higher amount of IFN compared to negative control cells or cells infected with either BVDV virus alone or BVDV/BRSV co-infection. Poly I:C stimulated cells synthesized IFN 11.3 times greater than negative control cells.

Cells co-infected with BRSV/NY93/c N-N^{pro} 18 EGFP had IFN levels 2.2 times greater than cells co-infected with BRSV/BVDV-wt, and 1.55 times greater than BRSV-infected cells ($p=0.0012$). Cells infected with BRSV had IFN level 1.75 times greater

than cells co-infected with BRSV/BVDV-wt (p=0.0012) and 2.6 times greater than cells infected with BVDV-wt alone, but 20% less than cells co-infected with BRSV/NY93/c N-N^{pro} 18 EGFP and equal to cells infected with NY93/c N-N^{pro} 18 EGFP alone (Figure 11).

DISCUSSION

Mutation of BVDV by inserting EGFP in the N^{pro} gene did not adversely affect BVDV replication. The parental, cloned, and mutated BVDV NY93 virus isolates replicated similarly in MDBK cells between 18 and 96 hours post-infection. While the mutated BVDV NY93 with EGFP inserted in N^{pro} protein (NY93/c N-N^{pro} 18 EGFP) replicated between 0.4 and 1.6 $TCID_{50}$ logs lower than the other two isolates, it still replicated well compared to the parental and cloned BVDV isolates with no significant differences.

Unlike cp BVDV, ncp BVDV infections *in vitro* and in the early bovine fetus antagonize type I IFN induction, whereas acute ncp BVDV infection of naïve cattle induced production of IFN (Brackenbury, 2005). Ncp BVDV N^{pro} mediates inhibition of type I IFN production in host cells (Gil, 2006; Schweizer, 2006). In this study, the parental and cloned BVDV NY93 virus isolates, ncp BVDV with intact N^{pro}, were poor inducers of type I IFN response in MDBK cells. In contrast, NY93/c N-N^{pro} 18 EGFP (a mutant with modified N^{pro} fused with EGFP) induced type I IFN response and cells infected with this virus produced IFN significantly greater than cells infected with the parental or cloned BVDV isolates. The increased production of type I IFN by NY93/c N-N^{pro} 18 EGFP may have reduced its replication, which suggests that the antagonism of type I IFN production enhances BVDV replication. These results are similar to a study

where recombinant type I IFN inhibited classical swine fever virus (CSFV) replication and other important viral pathogens in different homologous and heterologous cell lines (Xia, 2005).

Wild-type BVDV (BVDV-wt) and cloned BVDV NY93 (NY93/c) isolates strongly inhibited type I IFN production in MDBK cells stimulated with poly I:C, a synthetic dsRNA, 24 hours post-infection, while NY93/c N-N^{pro} 18 EGFP-infected cells produced IFN in significantly greater amounts than cells infected with the other viruses in this study. These results demonstrate the role of intact functional N^{pro} gene of BVDV in the antagonism of type I IFN production in host cells and suggest that NY93/c N-N^{pro} 18 EGFP is able to replicate effectively in the presence of type I IFN.

The parental (BVDV-wt) and NY93/c N-N^{pro} 18 EGFP virus isolates replicated to significantly different levels in BT cells, unlike MDBK cells in which BVDV-wt and NY93/c N-N^{pro} 18 EGFP replication was not significantly different. A similar observation was evident in the quantity of BVDV RNA in cell lysates. These results suggest that the influence of intact N^{pro} gene of BVDV on BVDV replication may vary depending on cell type. On the other hand, the BVDV-wt virus replicated similarly and had similar RNA levels in BT cells infected with BVDV-wt or BVDV-wt/BRSV showing that BRSV did not affect BVDV-wt replication. Also, NY93/c N-N^{pro} 18 EGFP replicated similarly and had similar RNA levels in BT cells infected with NY93/c N-N^{pro} 18 EGFP or NY93/c N-N^{pro} 18 EGFP/BRSV showing that BRSV did not affect BVDV replication even with modified N^{pro} region replication. Viral titration and real-time Q-RT-PCR, showed similar results and a similar pattern. There was a strong positive correlation between the amounts

of viral RNA and the infections virus ($TCID_{50}$) of each sample. A similar pattern in growth kinetics has been reported for NADL BVDV (Vassilev, 2000).

BRSV RNA levels in cells infected with BRSV, BRSV/BVDV-wt , or BRSV/NY93/c N-N^{pro} 18 EGFP were significantly greater than BRSV viral titers at all time-points post-infection. The differences between real-time Q-RT-PCR levels and viral titers could be due to the presence of noninfectious viral RNA or the over-expression of BRSV N protein in infected cells. These results are similar to a study, done previously in our lab, that showed that the viral titers and RNA levels of BRSV were significantly different at 1.5 to 72 hours post-infection (Achenbach, 2004). Similar to this study result, lower viral titers when compared to RNA levels using real-time Q-RT-PCR have been reported, with a strong correlation between the infectious virus titer and the amount of viral genome, for rift valley fever virus in cell culture and mouse sera (Garcia, 2001). Real-time Q-RT-PCR levels and viral titers of BRSV showed the same pattern of values. Correlation suggests a specific numerical relationship.

As in MDBK cells, ncp BVDV-wt with intact N^{pro} was a poor inducer of type I IFN response in BT cells, while NY93/c N-N^{pro} 18 EGFP (a mutant with modified N^{pro} fused with EGFP) induced type I IFN response and cells infected with this virus produced IFN significantly greater than cells infected with the BVDV-wt isolate. These results are consistent with a previous report for a mutant type 1 BVDV strain NADL with modified N^{pro} fused with EGFP, which showed reduced antagonism of type I IFN synthesis. They concluded that the type I IFN response was dependent on N^{pro} expression and independent of viral replication efficiency (Gil, 2006). The increased production of type I IFN by cells infected with NY93/c N-N^{pro} 18 EGFP may have reduced its replication to

significantly different levels, which suggests that the antagonism of type I IFN production enhances BVDV replication. These results are comparable to a study where recombinant type I IFN inhibited CSFV replication (Xia, 2005).

The nonstructural BRSV proteins, NS1 and NS2, together inhibit the phosphorylation and transcriptional activity of IRF-3 resisting type I IFN production (Bossert, 2002; Bossert, 2003). In this study, BRSV significantly inhibited IFN production in BT cells compared to positive control. Type I IFN inhibition was significantly greater in cells co-infected with BRSV/BVDV-wt than in cells co-infected with BRSV/NY93/c N-N^{pro} 18 EGFP or BRSV-infected cells. Type I IFN inhibition was similar in cells co-infected with BRSV/NY93/c N-N^{pro} 18 EGFP or BRSV-infected cells. These results suggest that NY93/c N-N^{pro} 18 EGFP, unlike BVDV-wt, does not enhance type I IFN inhibition by BRSV infection. *In vivo*, calves concurrently infected with BVDV and BRSV had more severe clinical signs of disease and extensive lung lesions, and shed virus in greater concentration for longer duration compared to calves infected with either virus alone. BVDV plays an indirect role by causing host immunosuppression while BRSV acts directly causing bronchopneumonia. BVDV and BRSV possibly act synergistically during co-infection of calves and as a result of synergism, respiratory and digestive tract diseases are enhanced (Brodersen, 1998). Increased BRSV replication in cells co-infected with BRSV/BVDV-wt compared to BRSV-infected cells may be due to BVDV-wt inhibition of type I IFN. The difference in BRSV replication was not seen between cells infected with BRSV alone and cells co-infected with BRSV/NY93/c N-N^{pro} 18 EGFP, and that may be due to the limited ability of NY93/c N-N^{pro} 18 EGFP to inhibit

type I IFN. These results suggest that NY93/c N-N^{pro} 18 EGFP, unlike BVDV-wt, does not enhance BRSV replication in BRSV co-infected cells.

In contrast to BVDV-wt, NY93/c N-N^{pro} 18 EGFP induces high levels of type I IFN, regardless of the type of bovine cell line used, showing the importance of the N^{pro} in antagonism of type I IFN in BVDV-infected cells. In addition, NY93/c N-N^{pro} 18 EGFP does not enhance BRSV infection during co-infection, unlike BRSV co-infection with BVDV-wt. Taken together, enhancement of BRSV by BVDV co-infection is mediated by IFN antagonism of BVDV N^{pro}. This engineered virus, NY93/c N-N^{pro} 18 EGFP, could be used as a BVDV live vaccine, but further studies are needed to determine the virulence of this mutant BVDV.

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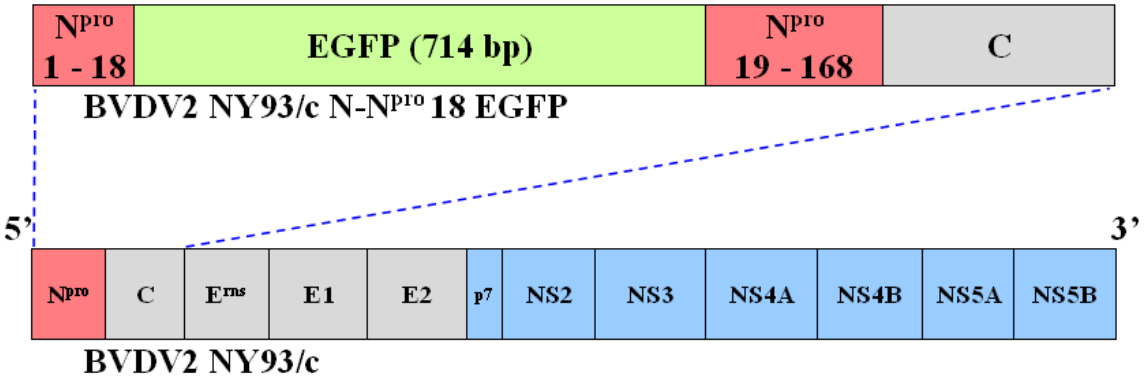


Figure 1: Genome structure of BVDV-NY93/c and BVDV-NY93/c N-N^{pro} 18 EGFP isolates.

Genome organization and encoded proteins of BVDV type II NY93/c isolate. The genome of BVDV NY93/c N-N^{pro} 18 EGFP isolate has the enhanced green fluorescent protein (EGFP) inserted between amino acids 18 and 19 of the N^{pro} protein.

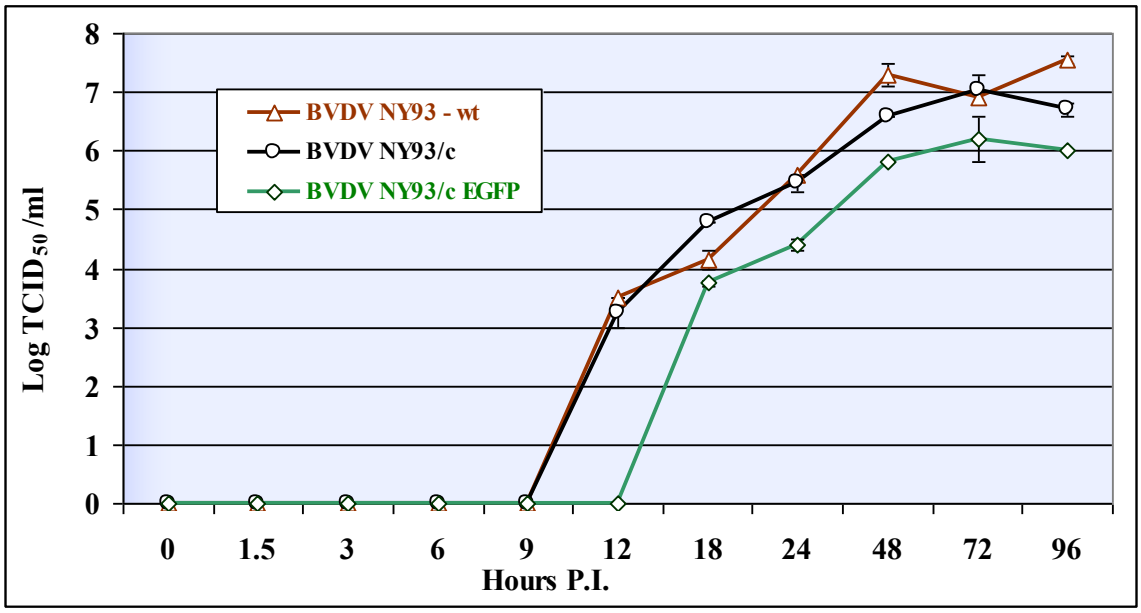


Figure 2: Growth kinetics of BVDV type II NY93 isolates, NY93-wt, NY93/c, and NY93/c N-N^{pro} 18 EGFP, in MDBK cells.

Madin-Darby bovine kidney (MDBK) cells were infected with NY93-wt, NY93/c, or NY93/c N-N^{pro} 18 EGFP isolates at an m.o.i. of 0.01 in subconfluent monolayers of cells in 6-well plates. Cell lysates were harvested at 0, 1.5, 3, 6, 9, 12, 18, 24, 48, 72, and 96 hours post-infection and assayed for viral infectivity using MDBK cells. Growth kinetics of these three BVDV isolates were compared. There were no significant differences between BVDV isolates at p=0.05. Bars represent ± standard error of the means (SEM). (Log $TCID_{50}$ /ml axis, 0 equates to < 2.3).

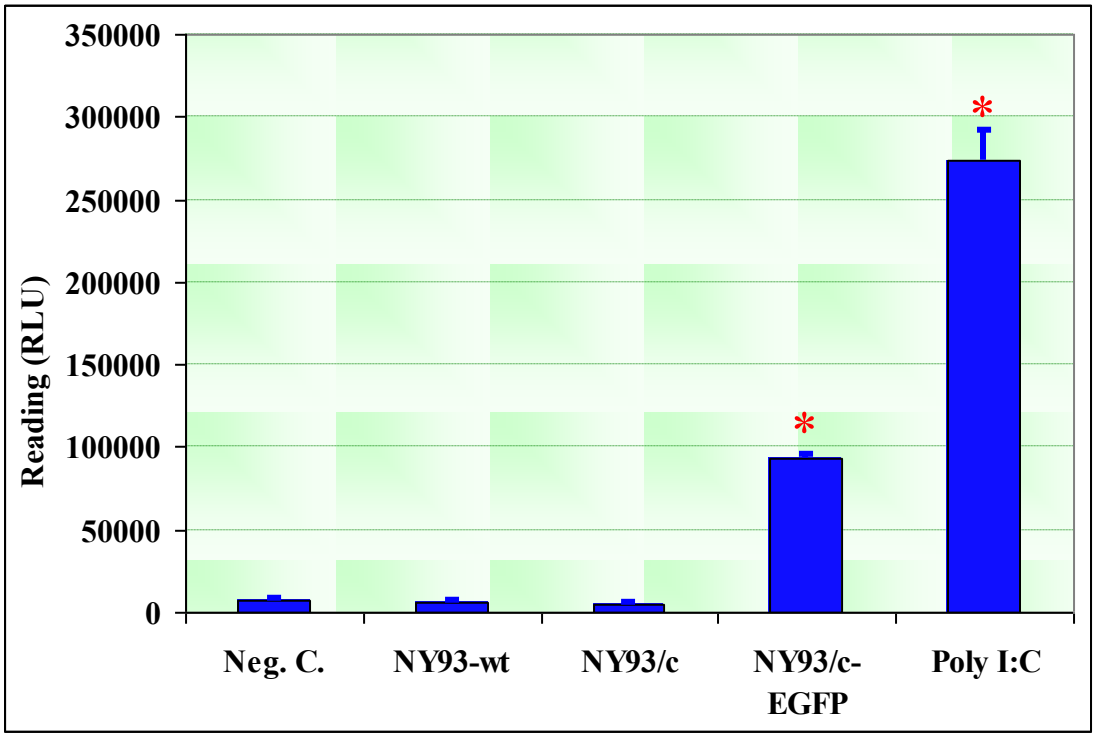


Figure 3: IFN responses of MDBK cells infected with BVDV type II NY93 isolates: NY93-wt, NY93/c, or NY93/c N-N^{pro} 18 EGFP.

Madin-Darby bovine kidney (MDBK) cells were infected with NY93-wt, NY93/c, or NY93/c N-N^{pro} 18 EGFP isolates at an m.o.i. of 0.5 in subconfluent monolayers of cells in 6-well plates. Cell lysates were harvested after 24 hours post-inoculation and clarified by centrifugation and pH adjusted to inactivate virus. Test samples were added to reporter cell plates containing NCL1-Luc-ISRE-Hygro cells and incubated for 8 hours. Reporter cell samples were lysed with passive lysis buffer and luciferase activity of cell lysates was measured using a luminometer. * Starred groups are statistically different from the other groups at p=0.05. Bars represent ± standard error of the means (SEM).

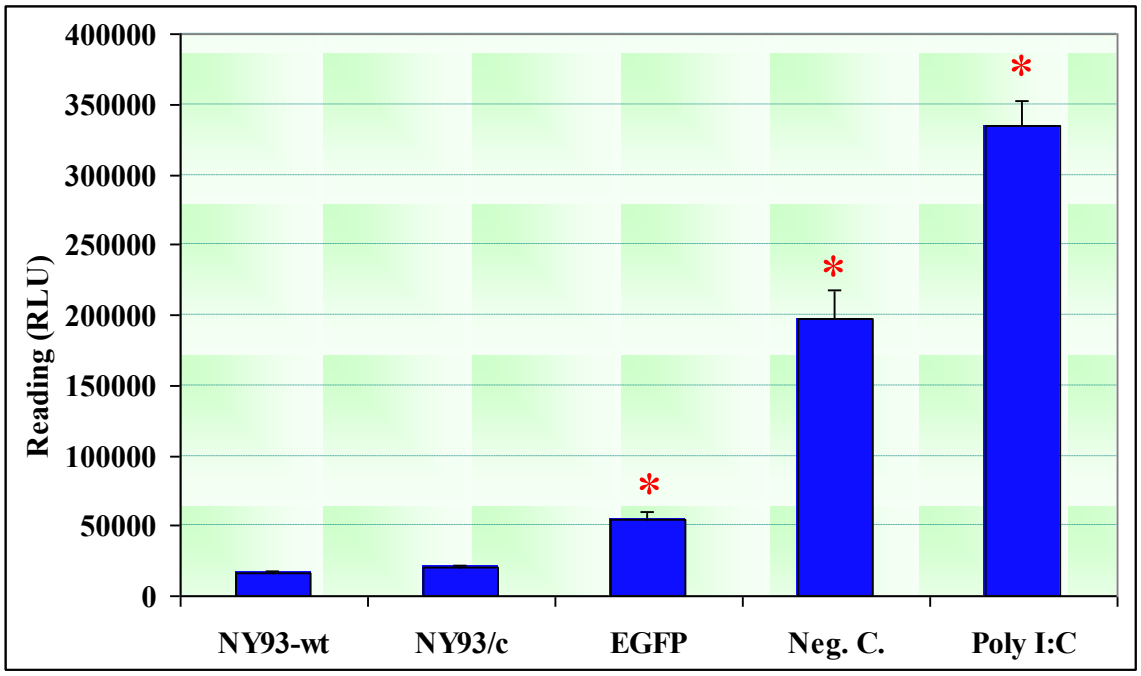


Figure 4: Inhibition of IFN synthesis of MDBK cells infected with BVDV type II NY93 isolates: NY93-wt, NY93/c, or NY93/c N-N^{pro} 18 EGFP.

Madin-Darby bovine kidney (MDBK) cells were infected with NY93-wt, NY93/c, or NY93/c N-N^{pro} 18 EGFP isolates at an m.o.i. of 1.2, plain media or poly I:C were added to MDBK in subconfluent monolayers of cells in 6-well plates and incubated. After 48 hours, the media and inoculum were changed and poly I:C added to all plates at a concentration of 50 µg/ml and cells were incubated for 20 hours. Cells then were harvested and clarified by centrifugation and pH adjusted to inactivate virus. Test samples were added to reporter cell plates containing NCL1-Luc-ISRE-Hygro cells and incubated for 8 hours. Reporter cell samples were lysed with passive lysis buffer and luciferase activity of cell lysates was measured using a luminometer. * Starred groups are statistically different from the other groups at p=0.05. Bars represent ± standard error of the means (SEM).

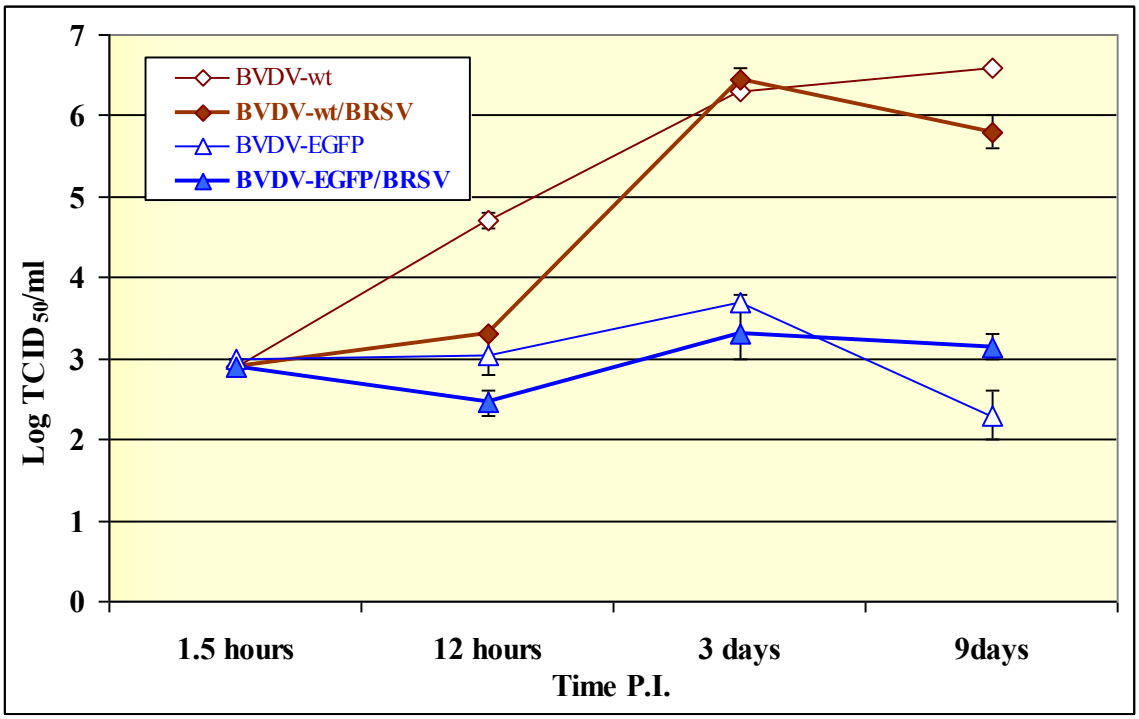


Figure 5: Growth kinetics of BVDV in BT cells infected with BVDV-wt, BVDV-wt /BRSV, BVDV-EGFP, or BVDV-EGFP/BRSV: testing the effects of BRSV on the growth kinetics of BVDV-wt and BVDV-EGFP isolates.

Bovine turbinate (BT) cells were infected with BVDV NY93 wild-type (BVDV-wt) alone, BVDV-wt/BRSV, BVDV NY93/c N-N^{pro} 18 EGFP (BVDV-EGFP), or BVDV-EGFP/BRSV at an m.o.i. of 0.6 in subconfluent monolayers of cells in 6-well plates. Cell lysates were harvested and assayed for BVDV infectivity at 1.5 hours, 12 hours, 3 days, and 9 days post-infection using MDBK cells. Growth kinetics were compared between cells inoculated with BVDV-wt alone or BVDV-wt/BRSV, and between cells inoculated with BVDV-EGFP alone or BVDV-EGFP/BRSV. There were no significant differences between the two inoculations of each pair at p=0.05. Bars represent ± standard error of the means (SEM).

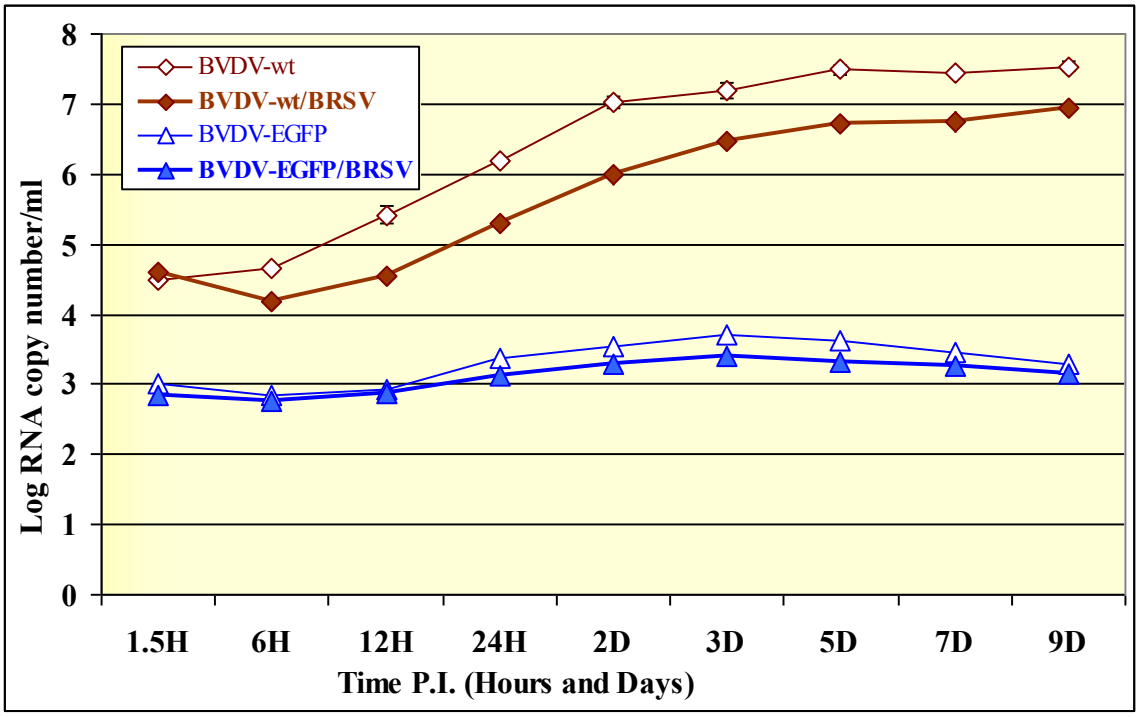


Figure 6: Comparison of BVDV RNA levels in BT cells infected with BVDV-wt, BVDV-wt/BRSV, BVDV-EGFP, or BVDV-EGFP/BRSV.

Bovine turbinate (BT) cells were infected with BVDV NY93 wild-type (BVDV-wt) alone, BVDV-wt/BRSV, BVDV NY93/c N-N^{pro} 18 EGFP (BVDV-EGFP) alone, or BVDV-EGFP/BRSV at an m.o.i. of 0.6 in subconfluent monolayers of cells in 6-well plates. Cell lysates were harvested at 1.5, 6, 12, and 24 hours, and 2, 3, 5, 7, and 9 days post-infection and BVDV RNA levels were quantified using Q-RT-PCR. RNA levels of BVDV were compared. There were no significant differences between the two inoculations of each pair at p=0.05. Bars represent ± standard error of the means (SEM).

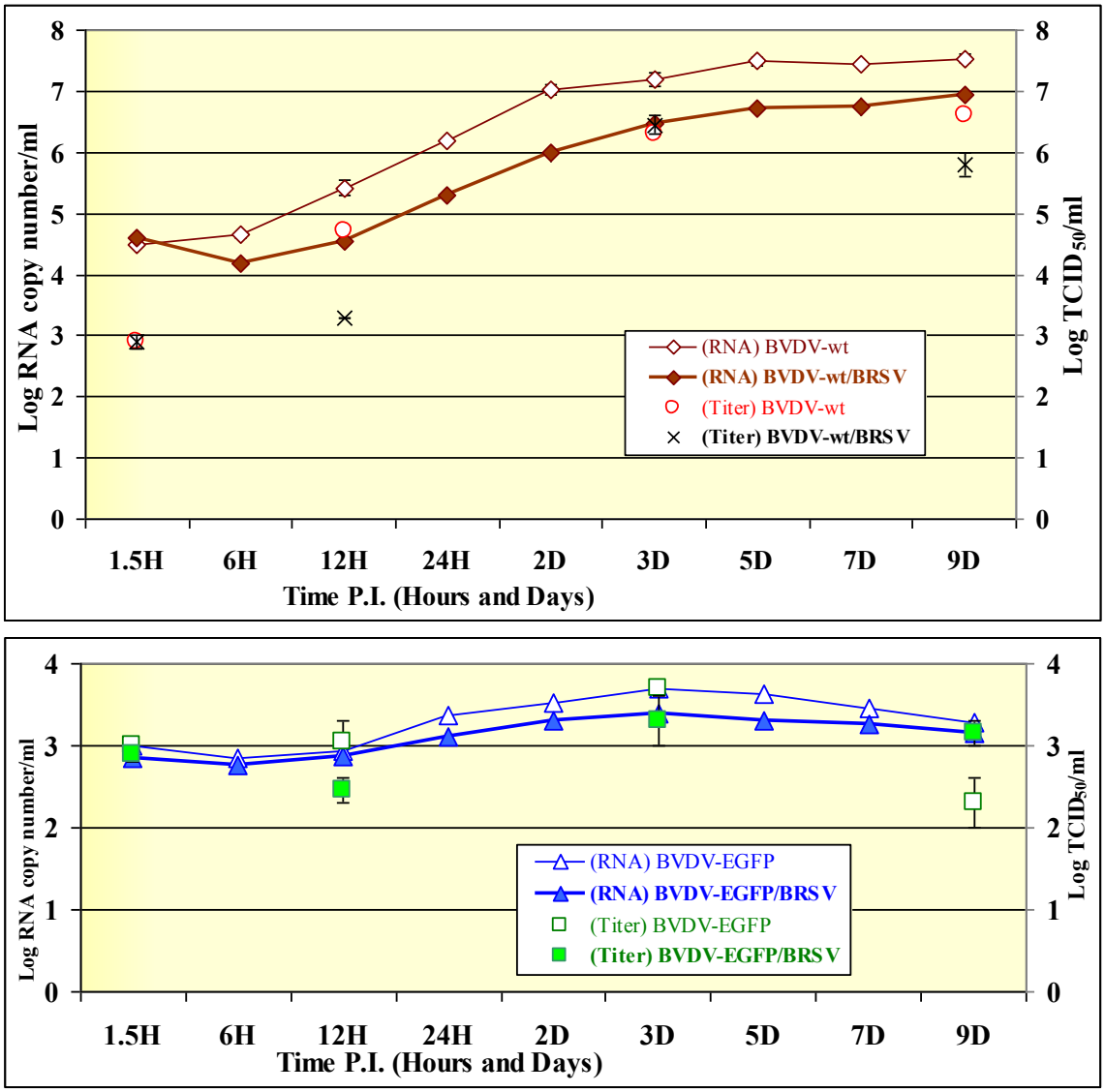


Figure 7: Comparison of BVDV RNA levels with BVDV titers (Log $TCID_{50}$/ml) in BT cells infected with BVDV-wt or BVDV-EGFP with or without BRSV.

Bovine turbinate (BT) cells were infected with BVDV NY93 wild-type (BVDV-wt) alone, BVDV-wt/BRSV, BVDV NY93/c N-N^{pro} 18 EGFP (BVDV-EGFP) alone, or BVDV-EGFP/BRSV at an m.o.i. of 0.6 in subconfluent monolayers of cells in 6-well plates. Cell lysates were harvested and BVDV RNA levels were quantified using Q-RT-PCR at 1.5, 6, 12, and 24 hours, and 2, 3, 5, 7, and 9 days post-infection (PI) and assayed for BVDV infectivity at 1.5 hours, 12 hours, 3 days, and 9 days PI. RNA levels of BVDV were compared to viral titers. There were no significant differences between the two methods at p=0.05. Bars represent ± standard error of the means (SEM).

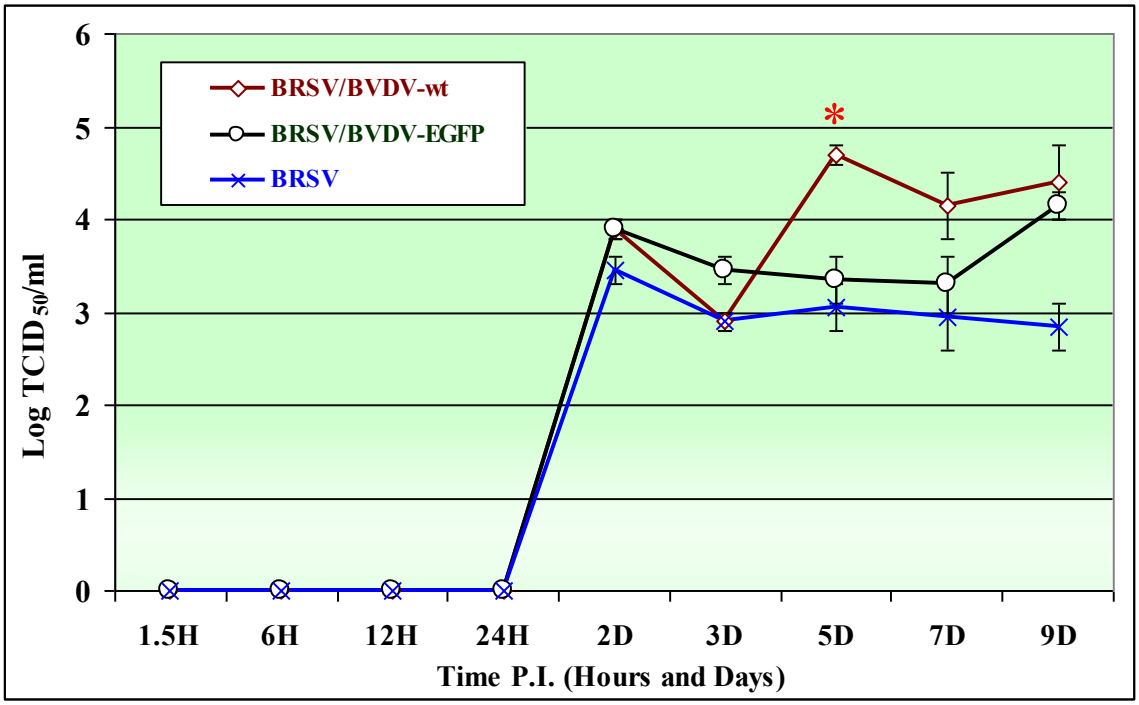


Figure 8: Growth kinetics of BRSV in BT cells infected with BRSV, BRSV/BVDV-wt, or BRSV/BVDV-EGFP: comparison of the effects of BVDV-wt and BVDV-EGFP isolates on the growth kinetics of BRSV.

Bovine turbinate (BT) cells were infected with BRSV 236-652 (BRSV) alone, BRSV/BVDV NY93 wild-type (BRSV/BVDV-wt), or BRSV/NY93/c N-N^{pro} 18 EGFP (BRSV/BVDV-EGFP) at an m.o.i. of 0.6 in subconfluent monolayers of cells in 6-well plates. Cell lysates were harvested at 1.5, 6, 12, and 24 hours, and 2, 3, 5, 7, and 9 days post-infection (PI) and assayed for BRSV infectivity using BT cells. Growth kinetics were compared. * Statistical significance ($p<0.05$) was evident on day 5 PI of cells inoculated with BRSV or BRSV/BVDV-EGFP when compared to cells inoculated with BRSV/BVDV-wt. Bars represent ± standard error of the means (SEM).

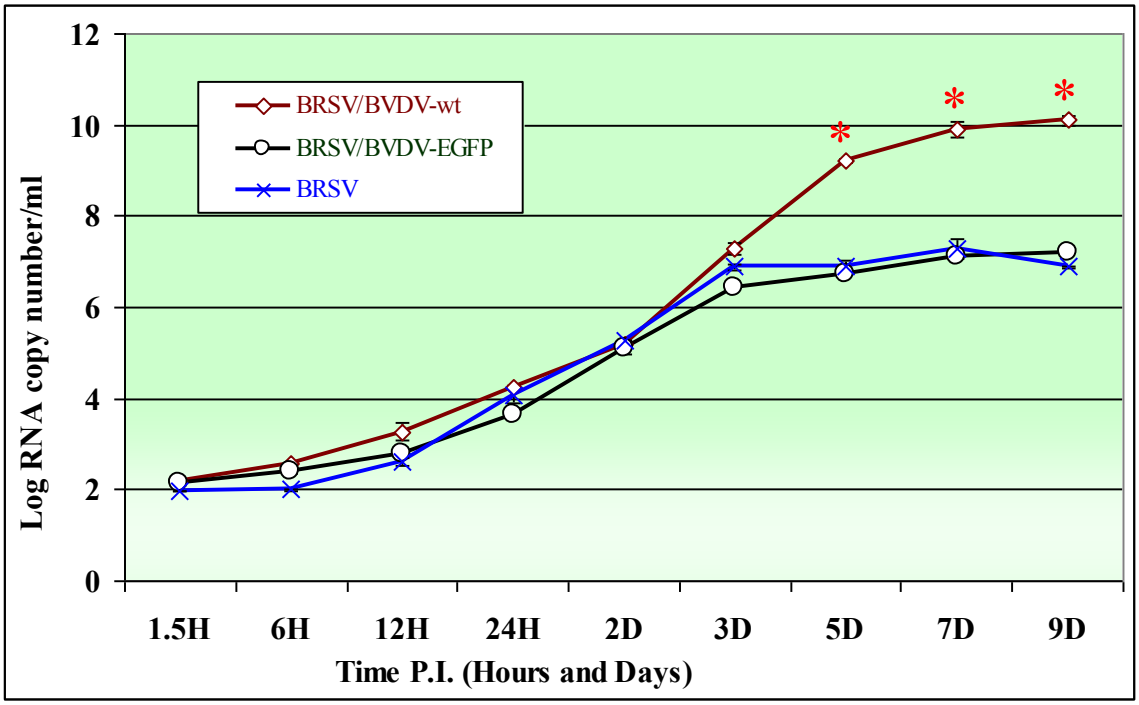


Figure 9: Comparison of BRSV RNA levels in BT cells infected with BRSV, BRSV/BVDV-wt, or BRSV/BVDV-EGFP: comparison of the effects of BVDV-wt and BVDV-EGFP isolates on the RNA levels of BRSV.

Bovine turbinate (BT) cells were infected with BRSV 236-652 (BRSV) alone, BRSV/BVDV NY93 wild-type (BRSV/BVDV-wt), or BRSV/NY93/c N-N^{pro} 18 EGFP (BRSV/BVDV-EGFP) at an m.o.i. of 0.6 in subconfluent monolayers of cells in 6-well plates. Cell lysates were harvested at 1.5, 6, 12, and 24 hours, and 2, 3, 5, 7, and 9 days post-infection (PI) and BRSV RNA levels were quantified using Q-RT-PCR. RNA levels of BRSV were compared. * Statistical significance ($p<0.05$) was present on days 5, 7, and 9 PI of cells inoculated with BRSV or BRSV/BVDV-EGFP when compared to cells inoculated with BRSV/BVDV-wt. Bars represent ± standard error of the means (SEM).

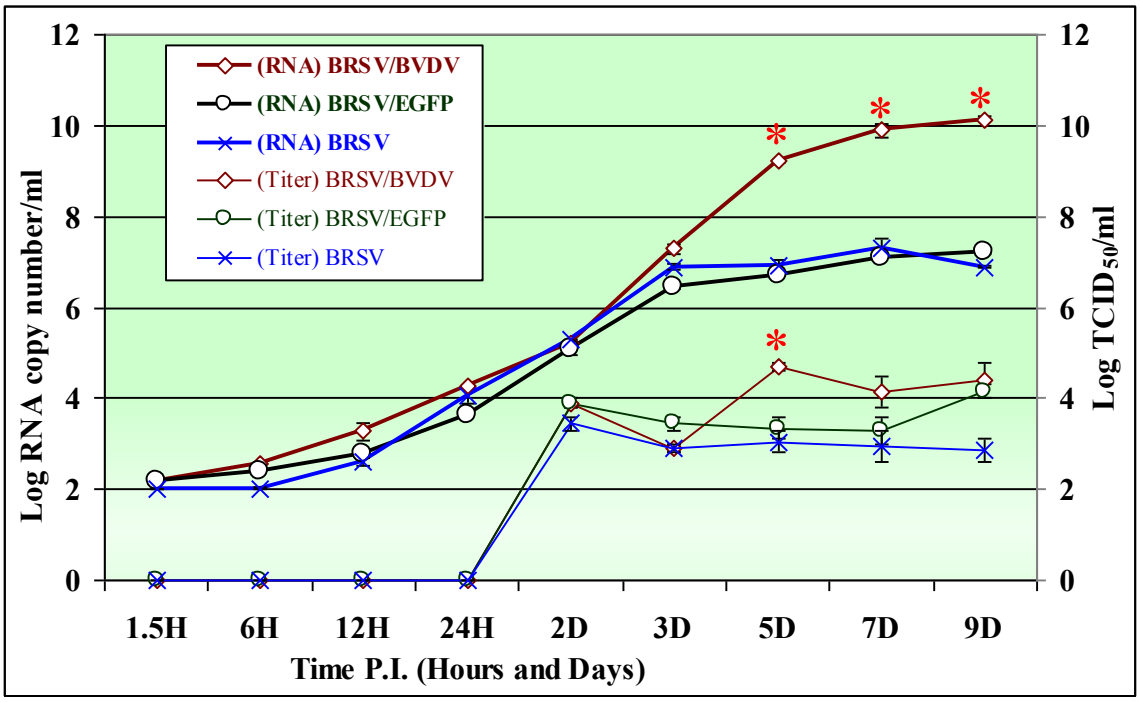


Figure 10: Comparison of BRSV RNA levels with BRSV titers (Log $TCID_{50}$/ml) in BT cells infected with BRSV, BRSV/BVDV-wt, or BRSV/BVDV-EGFP.

Bovine turbinate (BT) cells were infected with BRSV 236-652 (BRSV) alone, BRSV/BVDV NY93 wild-type (BRSV/BVDV-wt), or BRSV/NY93/c N-N^{pro} 18 EGFP (BRSV/BVDV-EGFP) at an m.o.i. of 0.6 in subconfluent monolayers of cells in 6-well plates. Cell lysates were harvested at 1.5, 6, 12, and 24 hours, and 2, 3, 5, 7, and 9 days post-infection (PI) and assayed for BRSV infectivity using BT cells and BRSV RNA levels were quantified using Q-RT-PCR. RNA levels of BRSV and BRSV titers were compared. These two methods showed the same pattern of values. Correlation suggests specific numerical relationship. * Statistical significance at p=0.05. Bars represent ± standard error of the means (SEM).

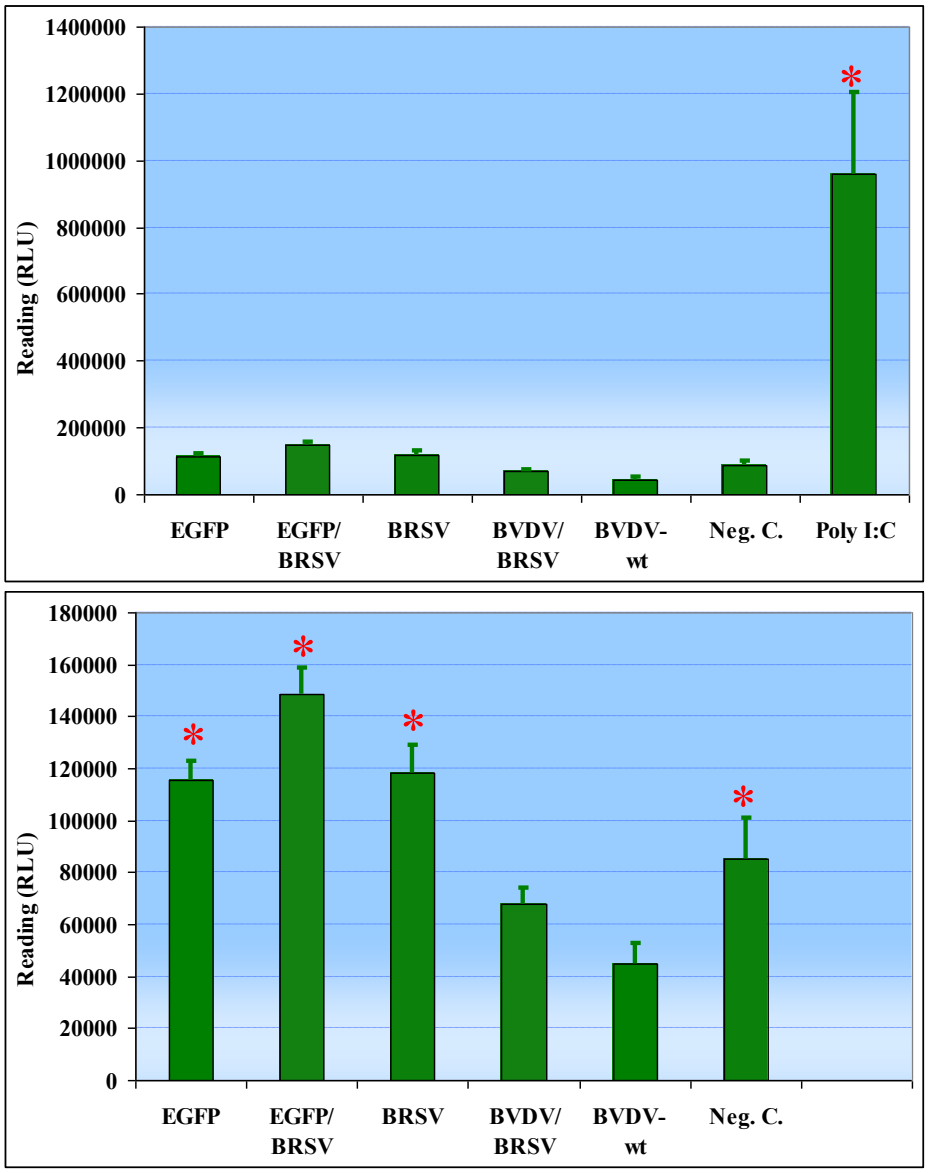
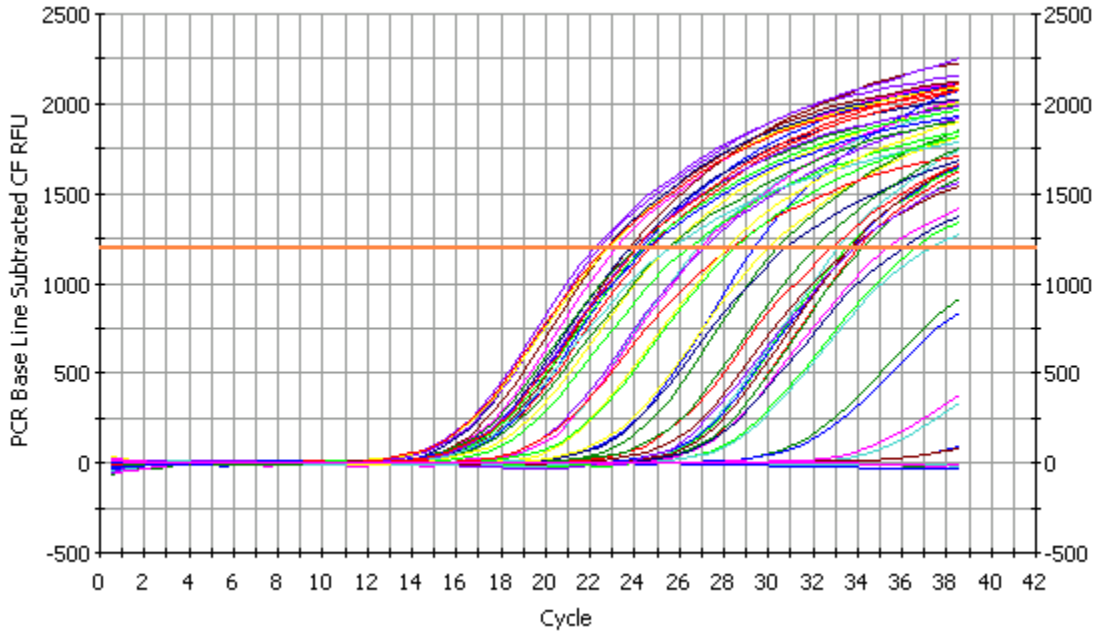


Figure 11: IFN responses of BT cells infected with BRSV, BVDV-wt, BVDV-wt /BRSV, BVDV-EGFP, or BVDV-EGFP/BRSV.

Bovine turbinate (BT) cells were infected with BRSV 236-652 (BRSV), BVDV NY93 wild-type (BVDV-wt), BVDV-wt/BRSV, BVDV NY93/c N-N^{pro} 18 EGFP (BVDV-EGFP), or (BVDV-EGFP/BRSV) at an m.o.i. of 0.6 in subconfluent monolayers of cells in 6-well plates and incubated for 48 hours. The media was changed with new media and cells were incubated for 20 hours. Cells then were harvested and clarified by centrifugation and pH adjusted to inactivate virus. Test samples were added to reporter cell plates containing NCL1-Luc-ISRE-Hygro cells and incubated for 8 hours. Reporter cell samples were lysed with passive lysis buffer and luciferase activity of cell lysates was measured using a luminometer. * Starred groups are statistically different from the other groups at p=0.05. Bars represent ± standard error of the means (SEM).

A.



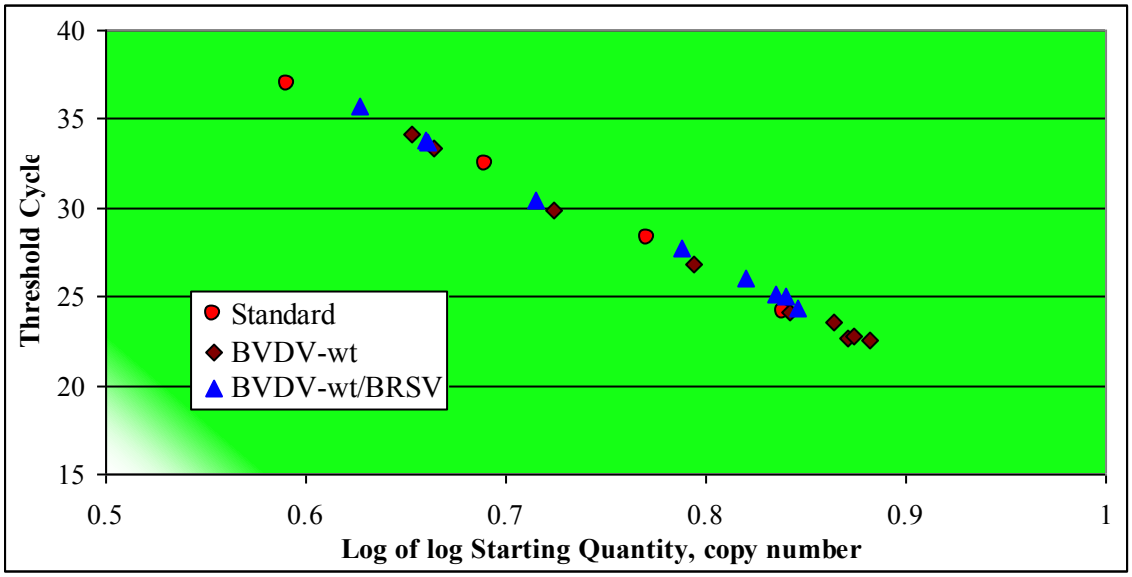
B.

post-infection	Ct of BVDV-wt alone		Ct of BVDV-wt/BRSV	
	Replication 1	Replication 2	Replication 1	Replication 2
1.5 hours	34.05	34.21	33.64	33.64
6 hours	33.53	33.13	35.39	36.06
12 hours	30.44	29.40	33.70	33.89
24 hours	26.80	26.97	30.87	30.03
2 days	24.36	23.81	27.12	28.31
3 days	23.22	23.92	25.50	26.46
5 days	22.87	22.47	24.72	25.51
7 days	22.71	22.88	24.47	25.60
9 days	22.29	22.75	24.15	24.63

- Ct = cycle threshold.

Figure 12: (**A**) The amplification plot and (**B**) cycle threshold values of real-time RT-PCR of BVDV NY93-wt samples with and without BRSV at each time-point. The y-axis represents the PCR base line subtracted RFU (relative fluorescence units). The x-axis represents cycle number.

C.

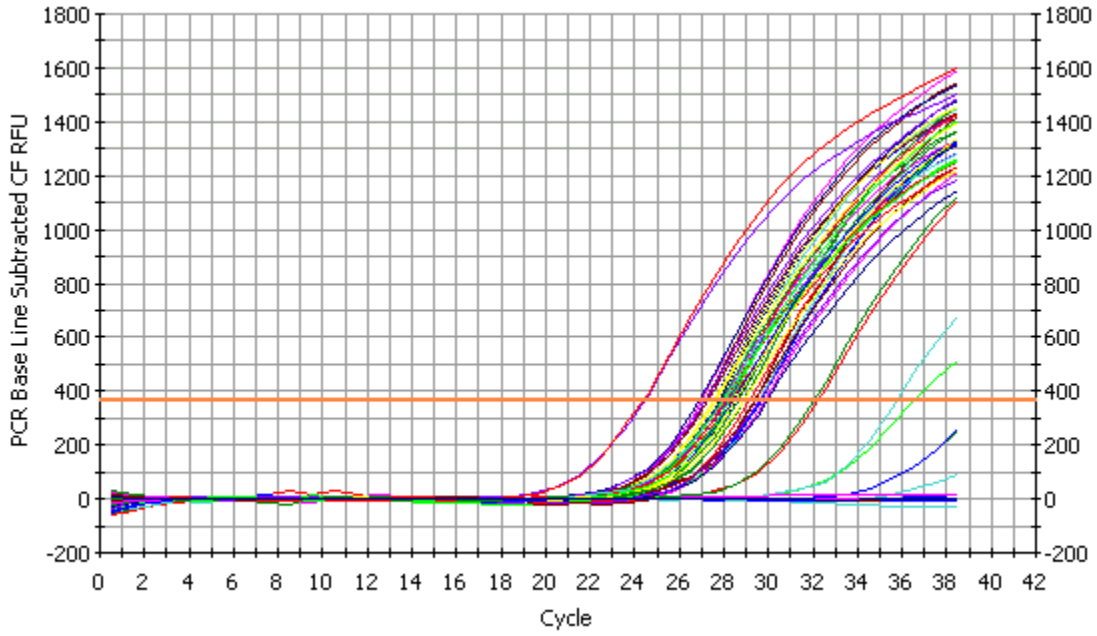


$Y = -51.444 (X) + 67.664$

Correlation Coefficient $R^2 = 0.997$

Figure 12C: A standard curve derived from amplification of serial ten-fold dilutions of known BVDV standard. Two wells for each dilution and two wells for each BVDV sample, from BVDV-wt or BVDV-wt/BRSV infected cell lysate from 1.5 hours to 9 days post-infection, were used.

A.



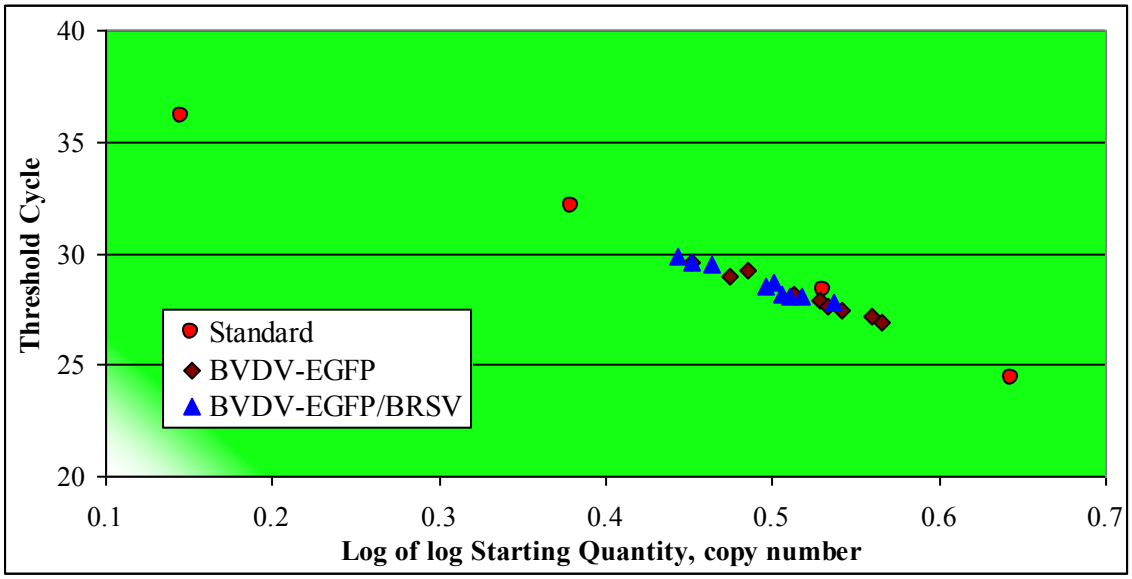
B.

post-infection	Ct of NY93/c N-N^{pro} 18 EGFP alone		Ct of NY93/c N-N^{pro} 18 EGFP /BRSV	
	Replication 1	Replication 2	Replication 1	Replication 2
1.5 hours	29.14	28.89	29.67	29.45
6 hours	29.65	29.60	29.84	29.96
12 hours	28.89	29.63	29.38	29.58
24 hours	27.88	27.91	28.51	28.88
2 days	27.56	27.27	28.11	28.08
3 days	26.99	26.89	27.66	28.02
5 days	27.16	27.16	28.30	27.86
7 days	27.77	27.56	28.42	27.94
9 days	28.24	28.06	28.62	28.43

- Ct = cycle threshold.

Figure 13: (**A**) The amplification plot and (**B**) cycle threshold values of real-time RT-PCR of BVDV NY93/c N-N^{pro} 18 EGFP samples with and without BRSV at each time-point. The y-axis represents the PCR base line subtracted RFU (relative fluorescence units). The x-axis represents cycle number.

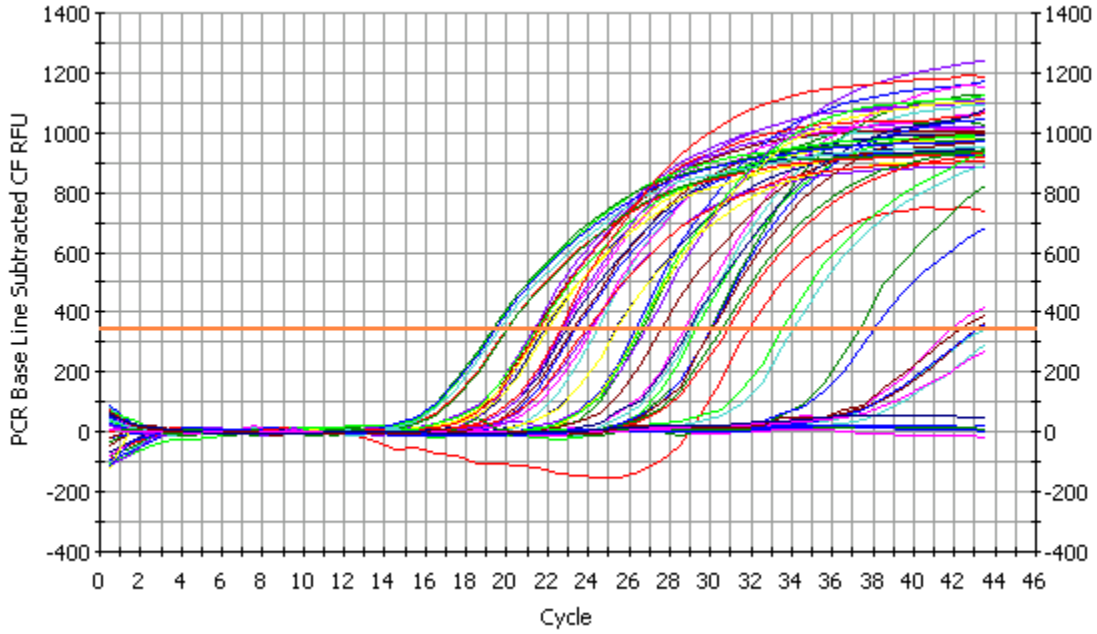
C.



Y = – 23.106 (X) + 40.082
Correlation Coefficient R^2 = 0.986

Figure 13C: A standard curve derived from amplification of serial ten-fold dilutions of known BVDV NY93/c N-N^{pro} 18 EGFP standard. Two wells for each dilution and two wells for each NY93/c N-N^{pro} 18 EGFP sample, from BVDV NY93/c N-N^{pro} 18 EGFP or NY93/c N-N^{pro} 18 EGFP /BRSV infected cell lysate from 1.5 hours to 9 days post-infection, were used.

A.



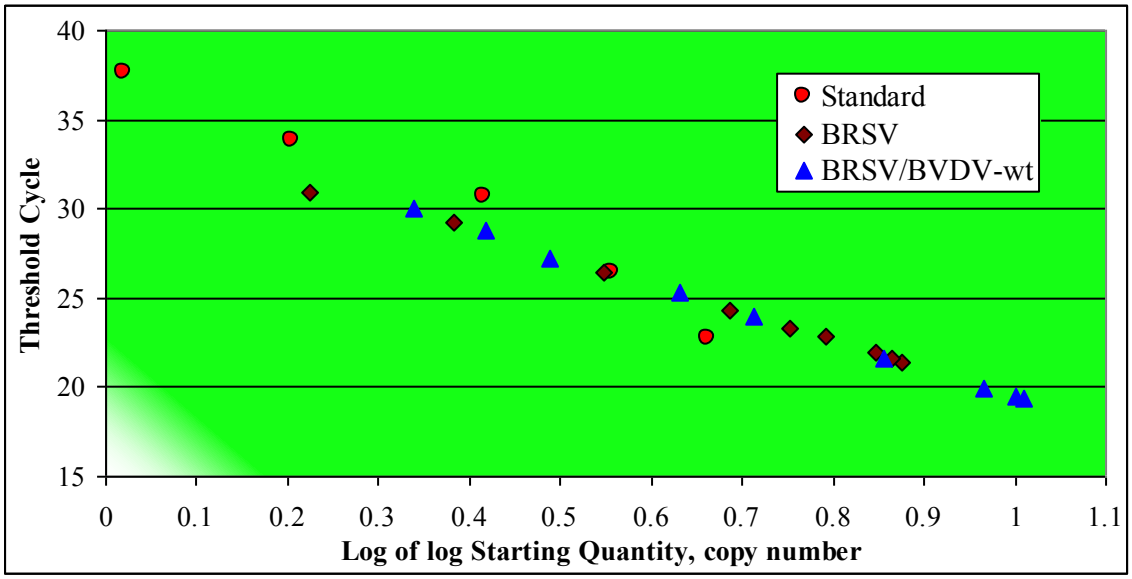
B.

post-infection	Ct of BRSV alone		Ct of BRSV/BVDV-wt	
	Replication 1	Replication 2	Replication 1	Replication 2
1.5 hours	31.84	29.99	30.00	30.09
6 hours	29.31	29.12	28.73	28.96
12 hours	26.66	26.32	27.60	26.80
24 hours	24.41	24.15	25.31	25.36
2 days	23.38	23.27	24.02	23.88
3 days	22.74	22.93	21.70	21.48
5 days	21.39	21.30	19.94	19.98
7 days	21.86	22.02	19.35	19.58
9 days	21.55	21.59	19.25	19.39

- Ct = cycle threshold.

Figure 14: (**A**) The amplification plot and (**B**) cycle threshold values of real-time RT-PCR of BRSV samples with and without BVDV-wt at each time-point. The y-axis represents the PCR base line subtracted RFU (relative fluorescence units). The x-axis represents cycle number.

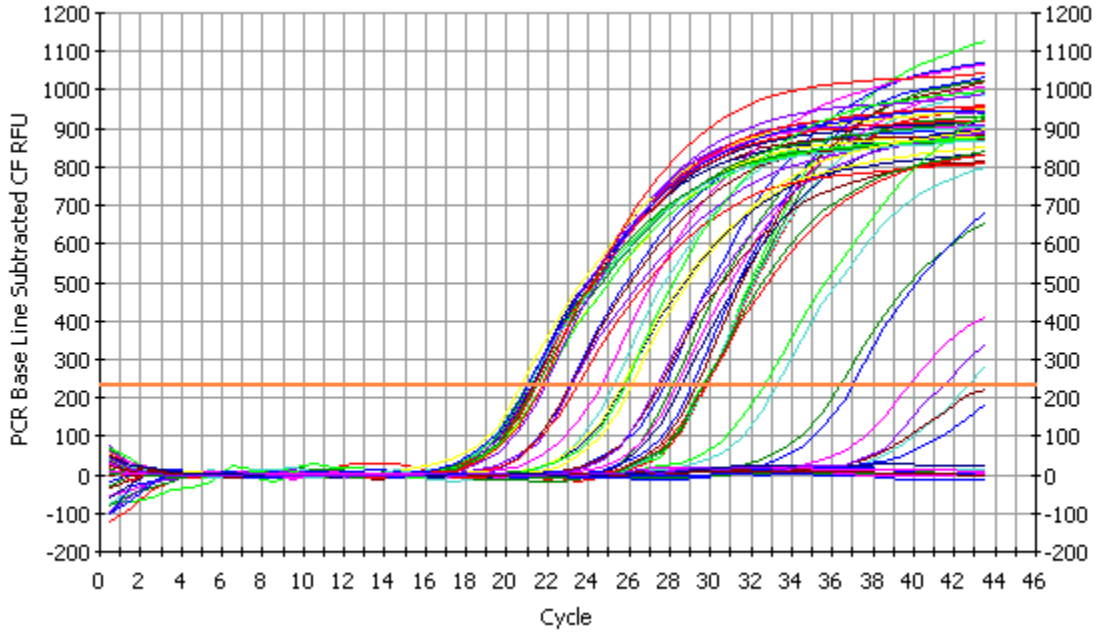
C.



$Y = -16.073 (X) + 35.457$
Correlation Coefficient $R^2 = 0.954$

Figure 14C: A standard curve derived from amplification of serial ten-fold dilutions of known BRSV standard. Two wells for each dilution and two wells for each BRSV sample, from BRSV or BRSV/BVDV-wt infected cell lysate from 1.5 hours to 9 days post-infection, were used.

A.



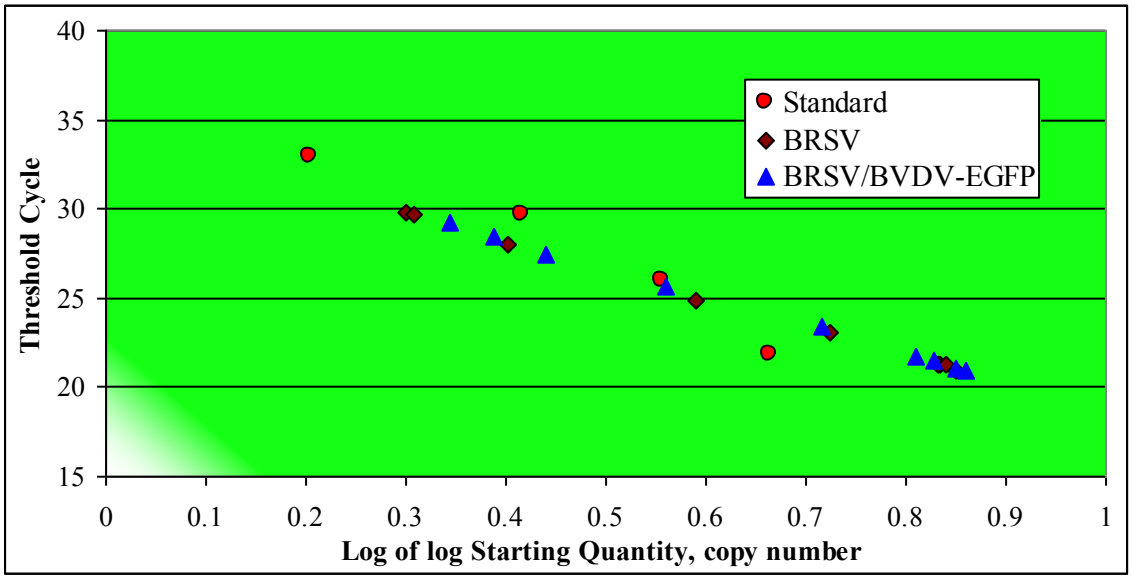
B.

post-infection	Ct of BRSV alone		Ct of BRSV/NY93/c N-N^{pro} 18 EGFP	
	Replication 1	Replication 2	Replication 1	Replication 2
1.5 hours	29.78	29.79	29.09	29.34
6 hours	29.65	29.78	28.37	28.60
12 hours	28.17	27.72	27.55	27.40
24 hours	25.20	24.61	25.67	25.62
2 days	23.08	23.11	23.22	23.53
3 days	21.35	21.23	21.74	21.75
5 days	21.37	21.13	21.44	21.46
7 days	21.09	20.69	21.09	21.05
9 days	21.27	21.31	20.95	20.98

- Ct = cycle threshold.

Figure 15: (**A**) The amplification plot and (**B**) cycle threshold values of real-time RT-PCR of BRSV samples with and without NY93/c N-N^{pro} 18 EGFP at each time-point. The y-axis represents the PCR base line subtracted RFU (relative fluorescence units). The x-axis represents cycle number.

C.



$Y = -15.770 (X) + 34.497$
Correlation Coefficient $R^2 = 0.95$

Figure 15C: A standard curve derived from amplification of serial ten-fold dilutions of known BRSV standard. Two wells for each dilution and two wells for each BRSV sample, from BRSV or BRSV/NY93/c N-N^{pro} 18 EGFP infected cell lysate from 1.5 hours to 9 days post-infection, were used.

APPENDIX I: MATERIALS

Cell Culture Solutions:

Dulbecco's Modified Eagle Medium (DMEM)-High Glucose

To prepare 6 liters:

ddH_2O	6 liters
DMEM	80.24 grams
$NaHCO_3$	22.2 grams

Adjust pH to 6.8 ~ 6.9 using 6 M HCl.
Filter sterilize with 0.2µm Supor® membrane filter into 0.5L bottles.
Use LB plates to test for bacteria and fungi.
Seal lids with parafilm and store at 4ºC.

CMF-PBS

To prepare 6 liters:

ddH_2O	6 liters
NaCl	48 grams
KCl	2.4 grams
$NaHCO_3$	5.25 grams
Glucose (Dextrose)	15 grams

Adjust pH to 7.2 using 6 M HCl.
Filter sterilize with 0.2µm Supor® membrane filter into 0.5L bottles.
Use LB plates to test for bacteria and fungi.
Seal lids with parafilm and store at 4ºC.

1X Trypsin-EDTA

0.5% Trypsin-EDTA.
To prepare a liter:

CMF-PBS	0.9 liter
10X Trypsin-EDTA	0.1 liter

Add phenol red to adjust color to light pink.
Filter sterilize with 0.2µm Supor® membrane filter into 100ml bottles.
Use LB plates to test for bacteria and fungi.
Store at – 20ºC.

Equine Serum

Hyclone, Logan, UT.

Fetal Bovine Serum

Hyclone, Logan, UT.

Freezing Media

Dimethyl Sulfoxide (DMSO)	8 ml
Glycerol	8 ml
Equine or fetal bovine serum	15 ml
DMEM	70 ml

Filter sterilize with 0.2µm Supor® membrane filter.
Store at 4ºC.

Luria-Bertani (LB) Media

To prepare a liter:

ddH_2O	1 liter
Bacto Tryptone	10 grams
Bacto Yeast Extract	5 grams
NaCl	10 grams

Adjust pH to 7.0 using 6 M HCl.
Pour into bottles.
Autoclave for 20 minutes.
Store at 4ºC.

Luria-Bertani (LB) Plates

To prepare a liter:

ddH_2O	1 liter
Bacto Tryptone	10 grams
Bacto Yeast Extract	5 grams
NaCl	10 grams

Adjust pH to 7.0 using 6 M HCl. Then add:

Bacto Agar	15 grams

Autoclave for 20 minutes.
Cool to 50ºC.

Add antibiotic if needed

Pour about 20 ml into each 90mm plate.
Leave plates uncovered in the laminar flow hood for 30 minutes to solidify.
Store at 4ºC.

Immunoperoxidase Staining Solutions:

0.01M PBS*

To prepare 6 liters:

ddH_2O	6 liters
Na_2HPO_4	12 grams
NaH_2PO_4	1.08 grams
NaCl	51 grams

Adjust pH to 7.6 using 6 M HCl.

Fixation Buffer (PBS/20% acetone)*

To prepare a liter:

PBS	0.8 liter
Acetone	0.2 liter

Binding Buffer*

To prepare a liter:

PBS	1 liter
NaCl	29.5 grams
Tween 20	0.1 ml

Wash Buffer*

To prepare 5 liters:

PBS	5 liters
Tween 20	2.5 ml

Substrate Buffer (Acetate Buffer 0.05M, pH 5.0)*

Prepare:

ddH_2O	200 ml	**Solution A**
Acetic acid	1.156 ml	

ddH_2O	500 ml	**Solution B**
Sodium acetate	6.8 grams	

Mix:

Solution A	148 ml	One liter of substrate buffer
Solution B	352 ml	
ddH_2O	500 ml	

Adjust pH to 5.0.

AEC Solution (3-Amino-9-Ethyl-Carbozole)*

To prepare a 4mg/ml stock solution:

N,N-dimethylformamide	100 ml
AEC	400 mg

Using aluminum foil, wrap bottle to protect from light.

* Store all the immunoperoxidase staining solutions at room temperature.

Agarose Gel Electrophoresis Solutions:

0.5X TBE (Working Solution)*

To prepare a liter:

5X TBE	100ml
ddH_2O	900ml

5X TBE Buffer*

(Tris base, Boric acid, and EDTA)

To prepare a liter of 5X TBE:

ddH_2O	1 liter
Tris base	54 grams
Boric acid	27.5 grams
0.5M EDTA (pH 8.0)	20 ml

Ethidium Bromide*

To prepare a 10mg/ml stock solution:

Sterile ddH_2O	10 ml
Ethidium bromide	100 mg

Using aluminum foil, wrap bottle to protect from light.

* Store all the agarose gel electrophoresis solutions at room temperature.

Interferon (IFN) Response Assay Solutions:

PBS with 0.1% BSA

To prepare 50ml of stock solution:

CMF-PBS	50 ml
Bovine Serum Albumin (BSA)	50 mg

Store at 4ºC.

(1mg BSA/ml PBS solution = 0.1% BSA solution)

1X Reporter Lysis Buffer with 1mg BSA/ml

Prepare a 1.25mg BSA/ml H_2O stock solution:

Sterile ddH_2O	40 ml
Bovine Serum Albumin (BSA)	50 mg

Store at 4ºC.

(1.25mg BSA/ml H_2O solution = 0.125% BSA solution)

To prepare a 1X reporter lysis buffer with 1mg BSA/ml solution:

1.25mg BSA/ml H_2O stock solution	2 ml
5X reporter lysis buffer	0.5 ml

Discard unused buffer after assay.

1X Passive Lysis Buffer

To prepare 5ml:

Sterile ddH_2O	4 ml
5X Passive Lysis Buffer	1 ml

Discard unused buffer after assay.

APPENDIX II: METHODS

Cell Culture Methods:

The following methods were used with three cell lines:

1. MDBK (Madin-Darby Bovine Kidney) cells:

Cell density is approximately 35 x 10^6 cells in a 100% confluent 75cm^2 flask, and 3 x 10^6 cells in a 100% confluent well of 6-well plate.

2. BT (Bovine Turbinate) cells:

Cell density is approximately 4 x 10^6 cells in a 100% confluent 75cm^2 flask, and 3 x 10^5 cells in a 100% confluent well of 6-well plate.

3. NCL1-ISRE-Luc-Hygro (modified bovine uterus) cells:

This reporter cell line has an ISRE (IFN Stimulated Response Element) gene coupled with a luciferase reporter gene and hygromycin antibiotic resistance gene. Use 6µl of Hygromycin B (50mg/ml solution) per ml of culture media to maintain selection. Cell density is approximately 6 x 10^6 cells in a 100% confluent 75cm^2 flask.

Establishing a Cell Culture from a Frozen Stock

- Mix 27ml DMEM with 3ml HS in a 75cm^2 tissue culture flask.
- Prevent ice crystal formation by thawing cell cryovial (ampule) rapidly in a 37ºC water-bath to prevent cell lysis.
- Add cells to tissue culture flask with media.
- Incubate at 37ºC with 5% CO_2. After 24 hours, change media.

Cell Culture Maintenance

- Discard the old media.
- Rinse cells twice with 10ml CMF-PBS.
- Add 5ml 0.05% trypsin-EDTA to cells, swirl, remove all but 1ml, and allow cells to separate for 3 minutes (8 minutes for MDBK cells).
- Tap flask to dislodge cells.
- Resuspend cells using 10ml of DMEM/10%HS.
- Add 0.5 – 3ml of cell suspension to 25ml 10%HS/DMEM/75cm^2 flask (35ml/162cm^2 flask).
- Incubate at 37ºC with 5% CO_2.
- Pass cells every week or at least twice a month.

Preparing a Frozen Stock of Cells

- Discard the old media.
- Rinse cells twice with 10ml CMF-PBS.
- Add 5ml 0.05% trypsin-EDTA to cells, swirl, remove all but 1ml, and allow cells to separate for 3 minutes (8 minutes for MDBK cells).
- Tap flask to dislodge cells.
- Resuspend cells in 5ml freezing media and pipette up and down.
- Aliquot each 1ml into a 2ml cryovial.
- Wrap in cotton, place in styrofoam container and freeze at – 80ºC.
- After 24 hours, place cryovials in a box at – 80ºC.

Testing for Bacteria and Fungi

To ensure that there is no contamination in materials, cells, and viruses, spread a 0.2ml sample onto an LB plate and incubate at 37ºC for bacteria and another 0.2ml sample onto another LB plate and incubate at room temperature for fungi, for two weeks.

Virus-work Methods:

Preparation of virus stocks

- Use 90% confluent MDBK-cell flasks for BVDV2 isolates. Use 90% confluent BT-cell flasks for BRSV236-652.
- Calculate the virus needed using the following formula:

$$\frac{\text{Cells per flask } \mathbf{X} \text{ \# of flask } \mathbf{X} \text{ \% confluency } \mathbf{X} \text{ m.o.i.}}{TCID_{50}}$$

- Add virus inoculum to 2%HS/DMEM to have a final volume of 5ml for each flask.
- Rinse flask twice with 10ml DMEM.
- Add 5ml virus inoculum/flask.
- Incubate at 37ºC with occasional rocking for 90 minutes.
- Add 20ml 2%HS/DMEM/75cm^2 flask (30ml/162cm^2 flask).
- Incubate at 37ºC with 5% CO_2 for 4 days for ncp BVDV2 isolates, and until CPE (Cytopathic Effects) for BRSV 236-652 (8-10 days).
- Freeze flasks at – 80ºC for at least 24 hours. Then thaw on ice.
- Pellet the virus if needed (see "Purified Pelleted Virus Stock"), or aliquot 0.5–2ml into 2ml cryovials, and store at – 80ºC.
- Titer the virus.

Purified Pelleted Virus Stock

- Using the infected flasks from previous protocol "Preparation of virus stocks".
- Thaw flasks on ice and transfer supernatant into 50ml conical tubes.
- Centrifuge for 30 minutes at 2,000 x g (~3,000 rpm) at 4ºC.
- Transfer supernatant into autoclaved ultracentrifuge tubes, making sure to transfer exactly the same amount into each balanced tube.
- Centrifuge for 2 hours at 25,000 rpm at 4ºC using Beckman Ultracentrifuge.
- Pour off supernatant carefully and put the tubes up side down on paper towels.
- Re-suspend viral pellet with 0.5ml DMEM, pipette up and down to mix.
- Use the same 0.5ml DMEM to re-suspend the second tube.
- Aliquot 100µl into four and 50µl into two 2ml cryovials.
- Store at – 80ºC.
- Titer the pelleted virus.

Virus Titration

1. Preparation of 96-well flat bottomed plate:

- Use MDBK cells for BVDV isolates, and BT cells for BRSV.
- Rinse flask twice with 10ml CMF-PBS. Add 5ml 0.05% trypsin-EDTA, swirl to cover cells and remove all but 1 ml.

- Allow cells to incubate at room temperature for 5 minutes, tap flask to dislodge cells.
- Re-suspend cells using 10ml of 3%HS/DMEM.
- Seed 2 x 10^4 MDBK cells/well, or 1 x 10^4 BT cells/well.
- Incubate at 37ºC with 5% CO_2 overnight.

2. Preparation of viral ten-fold serial dilution and inoculation of cells in plates:
- Prepare 5ml tubes with 0.9ml DMEM (or 0.45ml DMEM for Pelleted Virus Stock).
- Add 0.1ml stock virus into –1 tube (or 50µl pelleted virus stock), vortex.
- Transfer 0.1ml (or 50µl pelleted virus) from –1 to –2 tube, vortex, repeat from –2 to –3 tube, etc. Add 50µl of dilution –1 into each well of row #1, and 50µl of dilution –2 into row #2, etc.
- Add 50µl DMEM into each well of N.C. (Negative control) row.
- Incubate at 37ºC with 5% CO_2 for 4 days for BVDV, or 7 days for BRSV.
- Fix the plates.

Fixation
- Shake media off plates into a biohazard bag.
- Dry plates for 10–20 minutes in a hood.
- Add 0.1ml fixation buffer (PBS/20% acetone) per well.
- Incubate for 10–15 minutes at room temperature.
- Shake the fixation buffer off.
- Allow plates to dry overnight up side down.
- Detect viral antigen using Immunoperoxidase Staining.

Immunoperoxidase Staining (Table 1)
- Use fixed dry plates.
- Re-hydrate wells with 100µl binding buffer/well for 2-4 minutes.

1. Mab (primary monoclonal antibody) **:
- Use anti-BVDV Mab 348 for BVDV isolates, and 8G12 for BRSV.
- Make a 1:1000 dilution of Mab in binding buffer.
- Shake the binding buffer off plates.
- Add 50µl of the diluted Mab/well.
- Incubate at 37ºC for 1 hour.

2. Biotinylated Horse anti-mouse IgG (secondary antibody) **:
- Make a 1:200 dilution of IgG in binding buffer with 60% chicken serum.
- Shake the Mab off plates.
- Wash plate three times: Shake off.
  Add 100µl wash buffer/well.
  Shake off. Repeat three times.
  Drain on a towel.
- Add 50µl of the diluted IgG/well.
- Incubate at 37ºC for 1 hour.

3. HRP-Streptavidin**:

- Make a 1:500 dilution of HRP in binding buffer.
- Shake the IgG off plates.
- Wash plate three times with wash buffer.
- Add 50µl of the diluted HRP/well.
- Incubate at 37ºC for 1 hour.

Preparing a 3% H_2O_2 (Hydrogen peroxide) solution:
0.9ml substrate buffer + 0.1ml 30% H_2O_2
Use same day.

4. AEC (3-Amino-9-Ethyl-Carbozole) **:

- Use glassware with AEC always.
- For each 96-well plate, mix 5ml substrate buffer with 20µl 3% H_2O_2.
- Then add 0.3ml AEC slowly while swirling the mixture.
- Shake the HRP off plates.
- Wash plate three times with wash buffer.
- Add 50µl of the AEC/well. Incubate at room temperature for 2–4 minutes.
- When stained wells appear reddish brown color, shake off, rinse three times with tap water to stop the reaction.
- Let plate dry overnight, look for labeled viral antigen under the microscope and calculate the titer.

**See "table 1" for exact quantities/plate.

Growth Kinetics Assay

- Calculate the amount of virus of needed at an m.o.i. of 0.01 using the following formula:

$$\frac{\text{Cells per well } \mathbf{X} \text{ \# of wells } \mathbf{X} \text{ \% confluency } \mathbf{X} \text{ m.o.i.}}{TCID_{50}}$$

- Dilute viruses in DMEM to have a final volume of 1ml/well.
- Use 90% confluent MDBK cell 6-well plates for BVDV2 isolates.
- Use two plates for each time-point.
- Infect two wells with 1ml/well of BVDV2 NY93, BVDV2-NY93/c, or BVDV2-NY93/c-EGFP, or add DMEM as negative control.
- Incubate at 37ºC with occasional rocking for 90 minutes.
- Remove the inoculum and rinse with 1ml DMEM.
- Add 3ml 2%HS/DMEM/well
- Freeze the two plates of the first time-point (0 hour).
- Incubate the remaining time-points at 37ºC with 5% CO_2 for 1.5, 3, 6, 9, 12, 18, 24, 48, 72, 96 hours.
- Freeze plates of each time-point at – 80ºC for at least 24 hours.
- Thaw on ice.
- Aliquot 0.2–1ml into four 2ml cryovials and store at – 80ºC.
- Titer the viruses at each time-point.

Growth Kinetics of Concurrent Infections

- BVDV2-NY93 with BRSV (or BVDV2-NY93/c-EGFP with BRSV):
- Calculate the amount of virus needed at an m.o.i. of 0.6 for BVDV2-NY93, BVDV2-NY93/c N-N^{pro} 18 EGFP, and BRSV using the following formula:

$$\frac{\text{Cells per well } \mathbf{X} \text{ \# of wells } \mathbf{X} \text{ \% confluency } \mathbf{X} \text{ m.o.i.}}{TCID_{50}}$$

- Dilute viruses in 2%HS/DMEM to have a final volume of 1ml/well.
- Use 90% confluent BT cell 6-well plates.
- Use two plates for each time-point. Infect two wells with 1ml/well of BRSV, BVDV2-NY93 (or BVDV2-NY93/c-EGFP), or BVDV2-NY93 with BRSV (or BVDV2-NY93/c-EGFP with BRSV), or add 2%HS/DMEM as negative control.
- Incubate at 37ºC with occasional rocking for 90 minutes.
- Remove the inoculum and rinse with 1ml DMEM.
- Add 3ml 2%HS DMEM/well
- Incubate at 37ºC with 5% CO_2 for 1.5, 6, 12, 24, and 48 hours, and 3, 5, 7, and 9 days.
- Freeze plates of each time-point at – 80ºC for at least 24 hours.
- Thaw on ice and mix the two wells of each virus to have 6ml.
- Aliquot 0.2–3ml into five 2ml cryovials and store at – 80ºC.
- Titer the viruses at each time-point.
- Extract the mRNAs and perform real-time RT-PCR.

RT-PCR Methods:

RNA Extraction (Using Trizol® LS Reagent)

- Add 0.25ml of the sample (cell-culture supernatant that has virus) into a 1.5ml tube and add 0.75ml Trizol LS reagent (the volume ratio should be 1:3). Pipette up and down to mix and lyse the cells

  [Note:- Trizol LS reagent is very dangerous and harmful for eyes and skin].
- Incubate at room temperature for 5 minutes.
- Add 0.2ml chloroform into each tube.
- Cap tubes securely and shake vigorously (strongly) for 15 seconds.
- Incubate samples at room temperature for 15 minutes.
- Centrifuge samples at no more than 12,000 x g for 15 minutes at 2–8°C.
- Now, there are three phases: clear aqueous phase at the top, white phase (like a membrane) as inter-phase, and phenol phase at the bottom.
- Transfer the upper aqueous phase to a clean 1.5ml tube.
- Add 0.5ml isopropyl alcohol (Isopropanol).
- Incubate at room temperature for 10 minutes.
- Centrifuge at no more than 12,000 x g for 10 minutes at 2–8°C. The RNA pellet may not be visible.
- Pour off the supernatant carefully.
- Add 1ml 75% ethanol in DEPC-treated water (3 parts absolute ethanol + 1 part DEPC-treated water).
- Vortex samples and store at – 80°C.

RT-PCR Primers

For BVDV, the forward primer: 5' CAT GCC CAT AGT AGG AC 3'; and the reverse primer: 5' CCA TGT GCC ATG TAC AG 3' (Integrated DNA Technologies, Inc., Coralville, IA).

For EGFP, the forward primer: 5' GTG AGC AAG GGC GAG GAG CTG 3'; and the reverse primer: 5' CTT GTA CAG CTC GTC CAT GCC GAG AG 3' (Integrated DNA Technologies, Inc., Coralville, IA).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Pellet the RNA:

- Using the extracted RNA in 1ml 75% ethanol in DEPC.
- Centrifuge at 9,000 x g for 10 minutes at 2–8°C, and pour off the ethanol.
- Dry for 15 minutes in a speed-vac.
- Add 10µl of DEPC-treated water and pipette up and down.
- Incubate at 55°C for 15 minutes and on ice for a minute.

RT-PCR:

- Using thin-walled PCR tubes…

# of samples	one
Reverse primer (2pmol)	1 µl
10mM dNTP Mix	1 µl
DEPC-treated water	8 µl
RNA sample	2 µl
Final volume	12 µl

- Using PCR machine: perform the following cycle:

1 = 65°C	for 5:00 minutes
2 = 4.0°C	for 1:00 minute
3 = END	

- Add 8µl of the following mixture to each RNA sample:

# of samples	one
5X First strand Buffer	4 µl
0.1 M DTT	1 µl
RNase Inhibitor (10 U/µl)	2 µl
Superscript III RT	1 µl
From last step	12 µl
Final volume	20 µl

- Perform the following cycle:

1 = 56°C	for 1 hour
2 = 70°C	for 15:00 minutes
3 = 4°C	forever
4 = END	

- Now, the complementary DNA (cDNA) is formed.

- Add 2µl cDNA to 48µl of the following mixture:

# of samples	one
10X PCR Buffer (- $MgCl_2$)	5 µl
50mM $MgCl_2$	1.5 µl
10mM dNTP Mix	1 µl
Upstream primer (50pmol)	1 µl
Downstream primer (50pmol)	1 µl
Taq Polymerase	0.4 µl
DEPC-treated water	38.1 µl
cDNA	2 µl
Final volume	50 µl

- Perform the following cycle:

1 = 94°C	for 2 minutes
2 = 94°C	for 10 seconds
3 = 50°C	for 15 seconds
4 = 72°C	for 30 seconds
5 = Go to 2, 30 times	
6 = 72°C	for 10 minutes
7 = 4°C	forever
8 = END	

- Store at – 20°C, until performing an agarose gel electrophoresis.

Agarose Gel Electrophoresis

- To prepare a 1% agarose gel for a small gel apparatus, add 0.4g of agarose to 40ml of 0.5X TBE buffer.
- Microwave, cool, pour, and allow to solidify for 20 minutes.
- Mix 10µl of RNA samples or 1KB ladder with 2µl dye and load into wells of the gel.
- Run for approx. 90 minutes at 82V (voltage).
- Stain with 15µl ethidium bromide (10mg/ml) in 0.5X TBE buffer for 15 minutes.
- Destain in new 0.5X TBE buffer for 15 minutes.
- Visualize gel under ultraviolet light.

RNA Amplification Methods:

RNA Extraction

(Using QIAamp® Viral RNA Mini kit, spin protocol, Cat. # 52904, Qiagen, Valencia, CA)

- Pipette 560µl prepared buffer AVL (containing carrier RNA) into a 1.5ml tube.
- Add 140µl of the sample (cell-culture supernatant that has virus).
- Pulse-vortex for 15 seconds.
- Incubate at room temperature for at least 10 minutes, then briefly centrifuge.
- Add 560µl of absolute (96-100%) ethanol (for molecular biology use).
- Pulse-vortex for 15 seconds, then briefly centrifuge.
- Apply 630 µl of the solution into a QIAamp Mini spin column (in a 2ml tube), close, centrifuge at 6,000 x g (8,000 rpm) for 1 minute.
- Place the column into a new 2ml tube.
- Apply the remaining 630 µl of the solution into the column, and repeat the last step. [At this step, the RNA is binding to the silica-gel-based membrane of the column].
- Apply 500 µl of buffer AW1 (wash buffer), close, and centrifuge at 8,000 rpm for 1 minute to wash the contaminants away (first wash).
- Place the column into a new 2ml tube.
- Apply 500 µl of buffer AW2 (wash buffer), close, centrifuge at full speed (14,000 rpm) for 3 minutes to wash the contaminants away (second wash).
- Place the column into a new 1.5ml tube.
- Centrifuge at full speed for 1 minute.
- Place the column into a new 1.5ml tube.
- Open the column and apply 60 µl of AVE, close, incubate at room temp. for 1 minute, centrifuge at 8,000 rpm for 1 minute to elute the RNA from the membrane to the 1.5ml tube.
- Label the tubes and discard the columns.
- The RNA is stable for one year at – 20ºC or – 80ºC.
- Perform real-time RT-PCR.

Real-time RT-PCR Primers and Probe

For BVDV, the forward primer: 5'-GGGNAGTCGTCARTGGTTCG-3'; the reverse primer: 5'-GTGCCATGTACAGCAGAGWTTTT-3'; and probe: 5'-6-FAM-CCAYGTGGACGAGGGCAYGC-TAMRA-3'. The BVDV probe was labeled with a fluorescent reporter molecule (6-carboxy-fluorescein, 6-FAM) at the 5′ end and with a quencher molecule (6-carboxy-tetra-methyl-rhodamine, TAMRA) at the 3′ end. Primers and probe were based on sequences of the 5' UTR (Mahlum, 2002).

For BRSV, the forward primer: 5'-GCA-ATG-CTG-CAG-GAC-TAG-GTA-TAA-T-3'; the reverse primer: 5'-ACA-CTG-TAA-TTG-ATG-ACC-CCA-TTC-T-3'; and probe: 5'-/5HEX/-ACC-AAG-ACT-TGT-ATG-ATG-CTG-CCA-AAG-CA-/31ABkFQ/-3'. Based on conserved regions of the published sequences of the BRSV N gene (Boxus, 2005) (Integrated DNA Technologies, Inc., Coralville, IA).

Stock solutions of primers and probes are 100µm.

Real-Time RT-PCR (using BIO-RAD's iCycler iQ™)

- Prepare a table that shows which wells of the 96-well Real-time RT-PCR plate will be used. Use two wells for each RNA sample.
- Dilute the appropriate primers and probe of each virus in water.
- Prepare the master mix for the RNA of BVDV2:

# of reactions	Pre reaction
2X QuantiTect Probe RT-PCR Mix	12.5µl
Forward Primer (1:8 dilution)	1µl
Reverse Primer (1:8 dilution)	1µl
Probe (1:13.5 dilution)	1µl
QuantiTect RT Mix	0.25µl
RNase-Free Water	4.25µl
Final Volume	20µl

- Prepare the master mix for the RNA of BRSV 236-652:

# of reactions	Pre reaction
2X QuantiTect Probe RT-PCR Mix	12.5µl
Forward Primer (1:10 dilution)	1µl
Reverse Primer (1:10 dilution)	1µl
Probe (1:20 dilution)	1µl
QuantiTect RT Mix	0.25µl
RNase-Free Water	4.25µl
Final Volume	20µl

- Apply 20µl of the master mix into each well.
- Add 5µl of RNA sample.
- Using BIO-RAD's iCycler iQ™, Optical System Software:
- For the mRNA of BVDV2, define FAM as fluorophore and perform this cycle:

Cycle **1**	1X	One step	50ºC	For 30 min.
Cycle **2**	1X	One step	95ºC	For 15 min.
Cycle **3**	40X	Step **1**	95ºC	For 15 sec.
		Step **2**	55ºC	For 30 sec.
		Data collection and Real-time analysis enabled		
		Step **3**	72ºC	For 30 sec.
Cycle **4**	1X	One step	4ºC	HOLD

- For the mRNA of BRSV236-652, define HEX as fluorophore and perform this cycle:

Cycle **1**	1X	One step	50ºC	For 30 min.
Cycle **2**	1X	One step	95ºC	For 15 min.
Cycle **3**	45X	Step **1**	94ºC	For 15 sec.
		Step **2**	59ºC	For 60 sec.
		Data collection and Real-time analysis enabled		
Cycle **4**	1X	One step	4ºC	HOLD

- Copy the data and make a graph.

Interferon (IFN) response assay Methods:

Interferon Expression Assay

- BVDV2-NY93, BVDV2-NY93/c, and BVDV2-NY93/c-EGFP:
- Dilute viruses and poly I:C in 2%HS/DMEM to a final volume of 1ml/well.
- Use 10 wells of three 90% confluent MDBK cell 6-well plates.
- Infect two wells with 1ml/well of BVDV2-NY93, BVDV2-NY93/c, or BVDV2-NY93/c-EGFP, or add 2%HS/DMEM as negative control or poly I:C (100µg/ml) as positive control.
- Incubate at 37ºC with occasional rocking for 90 minutes.
- Add 2ml 2%HS/DMEM/well
- Incubate at 37ºC with 5% CO_2 for 24 hours.
- Freeze plates at – 80ºC for at least 24 hours.
- Thaw on ice and place the cell lysate in 15ml tubes.
- Centrifuge at 2,000 x g for 30 minutes at 4ºC, transfer to new 15ml tubes.
- Adjust pH to 2 with 2M HCl and test strips.
- Incubate at 4ºC for 24 hours to inactive the viruses.
- Adjust pH to 7 with 2M NaOH and test strips.
- Testing samples:
- Use 20 wells of three NCL1-ISRE-Luc-Hygro cell 12-well plates, which are seeded and incubated for 12 hours.
- Replace media of NCL1-ISRE-Luc-Hygro cell wells with 0.5ml of each sample, using two wells for each sample.
- Incubate at 37ºC with 5% CO_2 for 8 hours.
- Discard test sample, wash NCL1-ISRE-Luc-Hygro cells twice with 1ml CMF-PBS.
- Apply 100µl of 1X passive lysis buffer, and harvest the cells using scraper.
- Transfer cell debris and liquid into 1.5ml tubes, vortex for 15 seconds.
- Centrifuge at 12,000 x g at 4ºC for 2 minutes.
- Transfer the supernatant to new 1.5ml tubes.
- Store at – 80ºC for at least 24 hours.
- Perform luciferase assay.

Luciferase Assay

- Using samples in 1X passive lysis buffer.
- Use opaque luminometer (dark) 96-well plate.
- Apply 100µl of 1X lysis buffer with 0.01% BSA to 12 tubes (–1 to –12 tubes).
- Add 2.7µl of recombinant human IFN standard stock to 97.3µl of 1X lysis buffer with 0.01% BSA to have 100µl, vortex.
- Mixing the 100µl from last step with the 100µl of –1 tube, vortex, then transfer 100µl from –1 to –2 tubes until –12 tube (1:2 serial dilution).
- Equilibrate luciferase assay reagent and samples to room temperature.
- Leave the first two wells of the plate empty, and apply 20µl/well from each sample into two wells.
- Use FluoStar Optima luminometer, set to inject 100µl luciferase assay buffer/well, and measure luminescence.

Interferon Inhibition Assay of Concurrent Infection

- BVDV2-NY93 with BRSV & BVDV2-NY93/c-EGFP with BRSV:
- Dilute viruses and poly I:C in 2%HS/DMEM to a final volume of 1ml/well.
- Use 14 wells of four 90% confluent BT cell 6-well plates.
- Infect two wells with 1ml/well of BRSV, BVDV2-NY93, BVDV2-NY93 with BRSV, BVDV2-NY93/c-EGFP, or BVDV2-NY93/c-EGFP with BRSV, or add 2%HS/DMEM as negative control or poly I:C (100μg/ml) as positive control.
- Incubate at 37ºC with occasional rocking for 90 minutes.
- Add 2ml 2%HS/DMEM/well
- Incubate at 37ºC with 5% CO_2 for 48 (or 72) hours.
- Wash wells with 1ml DMEM, and replace with 3ml fresh 2%HS/DMEM/well for one well for each, and replace with 3ml fresh poly I:C in 2%HS/DMEM/well (50μg/ml) for the another well of each.
- Incubate at 37ºC with 5% CO_2 for 20 hours.
- Freeze plates at – 80ºC for at least 24 hours.
- Thaw on ice and place the cell lysate in 15ml tubes.
- Centrifuge at 2,000 x g for 30 minutes at 4ºC, transfer to new 15ml tubes.
- Adjust pH to 2 with 2M HCl and test strips.
- Incubate at 4ºC for 24 hours to inactive the viruses.
- Adjust pH to 7 with 2M NaOH and test strips. [Now, there are 14 different samples]
- Testing samples:
- Use 28 wells of four NCL1-ISRE-Luc-Hygro cell 12-well plates, which are seeded and incubated for 12 hours.
- Replace media of NCL1-ISRE-Luc-Hygro cell wells with 0.5ml of each sample, using two wells for each sample.
- Incubate at 37ºC with 5% CO_2 for 8 hours.
- Discard test sample, wash NCL1-ISRE-Luc-Hygro cells twice with 1ml CMF-PBS.
- Apply 100μl of 1X passive lysis buffer, and harvest the cells using scraper.
- Transfer cell debris and liquid into 1.5ml tubes, vortex for 15 seconds.
- Centrifuge at 12,000 x g at 4ºC for 2 minutes.
- Transfer the supernatant to new 1.5ml tubes.
- Store at – 80ºC for at least 24 hours.
- Perform luciferase assay.

Tabel 1: BVDV and BRSV isolation microtiter assay

Immunoperoxidase Staining for BVDV1 & 2
Enzyme-Linked Immunosorbent Assay (Elisa)

# of Plates		1	**2**	3	**4**	5	**6**	7	**8**	9	**10**
Mab*	Binding Buffer	6 ml	**10 ml**	15 ml	**20 ml**	25 ml	**30 ml**	35 ml	**40 ml**	45 ml	**50 ml**
	348[#] (or 8G12[¶])	6 µl	**10 µl**	15 µl	**20 µl**	25 µl	**30 µl**	35 µl	**40 µl**	45µl	**50 µl**
Biotinylated Horse α-mouse Ig	Binding Buffer	2 ml	**4 ml**	6 ml	**8 ml**	10 ml	**12 ml**	14 ml	**16 ml**	18ml	**20 ml**
	Chicken Serum	3 ml	**6 ml**	9 ml	**12 ml**	15 ml	**18 ml**	21 ml	**24 ml**	27 ml	**30 ml**
	Biotinylated horse α-mouse Ig	25 µl	**50 µl**	75 µl	**100µl**	125µl	**150µl**	175µl	**200µl**	225µl	**250µl**
HRP-Streptavidin	Binding Buffer	5 ml	**10 ml**	15 ml	**20 ml**	25 ml	**30 ml**	35 ml	**40 ml**	45 ml	**50 ml**
	HRP-Streptavidin	10 µl	**20 µl**	30 µl	**40 µl**	50 µl	**60 µl**	70 µl	**80 µl**	90 µl	**100µl**
AEC	Substrate Buffer	5 ml	**10 ml**	15 ml	**20 ml**	25 ml	**30 ml**	35 ml	**40 ml**	45 ml	**50 ml**
	3% H_2O_2 Solution	20 µl	**40 µl**	60 µl	**80 µl**	100 µl	**120µl**	140µl	**160µl**	180µl	**200µl**
	AEC Solution	0.3ml	**0.6ml**	0.9ml	**1.2ml**	1.5ml	**1.8ml**	2.1ml	**2.4ml**	2.7ml	**3 ml**

* Final dilution of monoclonal antibody (Mab) is 1:1000

[#] Primary Monoclonal antibody (Mab 348) directed against BVDV1 & BVDV2 E2 (gp53).

[¶] Primary Monoclonal antibody (Mab 8G12) directed against BRSV F protein.

APPENDIX III: BIBLIOGRAPHY

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