

AN ABSTRACT OF THE THESIS OF

Brian J. Zulauf for the degree of Master of Science in Veterinary Science presented on June 14th, 2007.

Title: Multiplex Real-Time PCR in the Detection and Differentiation of Bovine Respiratory Disease Pathogens

Abstract approved:

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Bovine respiratory syncytial virus (BRSV), bovine viral diarrhea (BVD) virus and bovine parainfluenza virus Type 3 (BPI3) virus are ubiquitous respiratory pathogens of cattle, which contribute to causation of bovine respiratory disease complex. As these respiratory viral pathogens cause very similar clinical signs, differential diagnosis of the pathogens is an important step in the proper treatment of the infected cattle. Therefore, a single tube fluorogenic multiplex real time-PCR-based TaqMan assay was developed for simultaneous detection of BRSV and BPI3V from bovine respiratory samples. TaqMan-PCR was optimized to quantify the viruses using the BioRad iCycler IQ real-time PCR detection system and dual-labeled fluorogenic probes. Primers and probes for our study were designed from a 100% conserved region of BRSV N gene and from BPI3V NP gene using GenBank sequences such that they can be used to differentiate BRSV and BPI3V in one-tube in clinical respiratory samples obtained from bovine species. Once the technique was optimized with validated reference strains and field isolates, the assay was applied to routine diagnostic samples. Respiratory samples collected from 100 cattle across the state of Oregon were screened for BRSV and BPI3V viruses by real time-PCR and virus isolation. The monoplex real-time PCR identified 19 samples positive for BRSV, 11 for BPI3V and 14 for BVD. The multiplex real-time PCR identified 17 samples positive for BRSV and 4 samples positive for BPI3V. Among positive samples, 2 samples showed positive for both BRSV and BPI3V. BVDV was left out of the multiplex reaction due to primer interference. In conclusion, the multiplex real-time TaqMan-PCR described here

for detection and quantitation of BRSV and BPI3V viruses has been shown to be sensitive and specific. Rapid turnaround time, reproducibility and ease of use make this technique a valuable diagnostic tool for detection of BRSV and BPI3V in individual or pooled respiratory samples.

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Multiplex Real-Time PCR in the Detection and Differentiation of Bovine Respiratory
Disease Pathogens

by
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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented June 14, 2007
Commencement June 2008

Master of Science thesis of Brian J. Zulauf presented on June 14th, 2007.

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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ACKNOWLEDGEMENTS

I would like to express sincere appreciation to Dr. Manoj Pastey for his support from the very beginning of this process. Without his help, this never would have been possible. In addition, to Rocky Baker in the OSU Veterinary Diagnostic Lab and everyone who works there, a large debt is owed for their patience and unbelievable willingness to drop what they are doing to help. Thank you to Dr. Dan Rockey for helping to keep everything on track. Thank you to my wonderful graduate committee (Jean Hall, Ling Jin and Becky Donatelle) for taking the time to allow this journey to come to completion. And finally thank you to my parents for always giving me the support I need when I needed it and to K Double Sizzle for always being there when I needed someone to talk to when times were good and bad.

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Multiplex Real-Time PCR in the Detection and Differentiation of Bovine Respiratory Disease Pathogens

Introduction

Respiratory diseases have had a major impact on the overall health of cattle and continue to be of great importance even today. Many of the diseases that have been shown to impact the respiratory tract of cattle have been grouped into an overall category known as bovine respiratory disease (BRD) complex. This includes shipping fever syndrome, mucosal disease, enzootic calf pneumonia, acute respiratory distress syndrome, hemorrhagic syndrome, and atypical interstitial pneumonia (Baker 1995; Ames 1997; Apley 2006). Pathogens that have been implicated in the causation of this complex include microbial and viral pathogens. In terms of microbial pathogens, the most common are *Mannheimia haemolytica*, *Histophilus somni* and *Pasteurella multocida* (Apley 2006). Studies have shown that the major viral pathogens that contribute to BRD are: Infectious bovine rhinotracheitis (IBR), bovine parainfluenza – 3 virus (BPI-3), bovine viral diarrhea virus (BVDV), bovine adenovirus (BAV) and bovine respiratory syncytial virus (BRSV) (Lehmkuhl and Gough 1977; Gagea, Bateman et al. 2006). These pathogens, along with stress and other environmental factors, have been shown to have a synergistic effect on each other so that the severity of the disease is worse with concurrent infections than with an individual pathogen (Brodersen and Kelling 1998). In addition, viral pathogens have been shown to weaken the host's immune response making the host more susceptible to opportunistic pathogens (Potgieter 1995).

Although there is a large list of pathogens that contribute to BRD, the clinical signs of infection are very similar. Typical signs include rapid respiration, anorexia, nasal and/or ocular discharge, depression, fever, interstitial pneumonia and reproductive failure (Brock 2004; Solis-Calderon, Segura-Correa et al. 2005; Apley 2006). Because of the similarities in clinical presentation, it is important to develop methods to quickly and accurately differentiate the cause of infection so that the appropriate treatment can be started and the infected calves can be appropriately quarantined. Current treatments for BRD include anti-microbial therapy (Apley 1997), ancillary therapy (Apley 1997) and vaccinations against the most common viral pathogens (Kucera CJ 1983; Lindberg and Alenius 1999; Pennathur, Haller et al. 2003).

Viral pathogens create a unique challenge in that the most common weapon at a veterinarian's disposal is vaccination, but the impact of the various viral agents on BRD remains uncertain. It is important to get a sense of the exact prevalence of each pathogen in order to better understand what vaccination strategy is best suited for the health and well being of the herd. In addition, in the case of BVDV, many calves may develop what is described as a persistent infection where they are asymptomatic but shed copious amounts of viral particles, thus infecting many other calves (Ames 1986). It is for this reason that it is important to search for new and improved methods for detecting BRD pathogens that are more specific, reliable and rapid than current options that are available. Rapid detection of viral infections and a good understanding of viral pathogenesis are crucial in order to control the spread of disease, for the development of antiviral drugs, and for biodefense. Earlier detection of BRD would enable a more targeted treatment regime and earlier isolation of infected individuals.

The diagnostic arsenal at a veterinarian's disposal has increased significantly over the years and now includes virus isolation, histopathologic examination, enzyme-linked immunosorbent serum assay (ELISA), and reverse transcription polymerase chain reaction (RT-PCR) procedures, among others (West, Bogdan et al. 1998). Of the many options available, the two methods that have gained the widest acceptance are ELISA and RT-PCR (West, Bogdan et al. 1998; Saliki and Dubovi 2004). While ELISA is undeniably sensitive and specific (Hazari, Panda et al. 2002), they require the use of viral protein specific antibodies for detection of the virus. RT-PCR has been used for the detection of both BRSV (Valarcher, Bourhy et al. 1999; Almeida, Domingues et al. 2004) and BVDV (Bhudevi and Weinstock 2001; Deregt, Carman et al. 2002; Mahlum, Haugerud et al. 2002) and has proven to be more sensitive and rapid than the methods listed above. However, monospecific RT-PCR are expensive and resource intensive because they require separate amplification of each target followed by identification of the target by PCR fragment size on electrophoresis or hybridization with probes post PCR. Multiplex real-time PCR assays have a number of advantages over RT-PCR, including improved sensitivity, decreased cost, smaller sample size, and decreased time. Thus they are more suitable for high throughput.

In this study, a multiplex real-time PCR assay was investigated that would be able to simultaneously screen for BRSV, BVDV and BPI-3. The primers and probes used in this study were designed from 100% conserved regions of the BRSV and BPI-3 nucleocapsid genes. In addition, the primers and probes for the detection of BVDV were designed based on the 126-strain nucleotide alignment that amplifies 190 bases of a 5' untranslated region (UTR) of the genome. The purpose of this study was to show that multiplex real-time PCR is a rapid and efficient method for the detection and differentiation of multiple viral pathogens and that it can be an invaluable tool in the diagnosis and treatment of the BRD complex.

Review of Literature

Bovine Respiratory Disease

In terms of impact, both financially and in overall health, bovine respiratory disease (BRD) complex has been the most important contributor to losses in the beef and dairy cattle industries for many years, because of increased mortality, the cost of metaphylactic and therapeutic uses of antibiotics, and reduced growth performance of affected cattle (Gagea, Bateman et al. 2006). Calves are most often affected by BRD because of the waning influence of their mother's immune system, and their own still relatively naïve immune system. Many factors influence the onset of BRD in calves; some of the most important are the interactions between a wide range of pathogens, the calf's immune status, and environmental stresses (Godinho, Sarasola et al. 2007). Physical respiratory defenses (filtration, removal and adhesion resistance) can be compromised by inhaled noxious gases, temperature extremes, dehydration and concurrent viral infections resulting in damage to the mucosal lining of the upper respiratory tract and increased viscosity of respiratory secretions (Ames 1997). An important component of BRD in dairy calves is pneumonia or enzootic calf pneumonia. Shipping fever, acute respiratory distress syndrome and atypical interstitial pneumonia are also included in the bovine respiratory disease complex. Pneumonia is traditionally described as affecting calves from 2-6 months of age, but can affect calves as young as 2 weeks, with a peak occurrence at 5-6 weeks (Ames 1997). A serious challenge clinicians must face when dealing with BRD is the large list of pathogens that are able to cause similar signs that are all grouped together as BRD. Typically BRD involves both bacterial and viral infections, however therapeutic efforts are often primarily aimed at bacterial pathogens (Apley and Fajt 1998). BRD pathogens have been shown to cause cattle to become more susceptible to infection by other pathogens. BRD viral pathogens have, in fact, even been shown to have a synergistic effect on each other. In a study done in 1998, 9 to 12-month-old calves were infected individually with either bovine respiratory syncytial virus (BRSV) or bovine viral diarrhoea virus (BVDV) or concurrently with both. The calves infected with both viral pathogens developed more severe clinical signs of disease (fever and diarrhoea), and leukopenia. They also shed the virus from nasal secretions in

greater concentration and for a longer duration in addition to having more extensive lung lesions (Brodersen and Kelling 1998). It was shown in a study in 1985 that BRSV, bovine parainfluenza virus-3 (BPI-3) and bovine herpes virus-1 (BHV-1) have a direct impact on cellular defenses, including alveolar macrophages and neutrophils (Liggitt 1985). A different study in 1995 found evidence that BVDV may even impair pulmonary intravascular macrophages and neutrophils (Potgieter 1995). These studies seem to indicate a possible mechanism in which infection with a particular virus can leave a calf more susceptible to infection by another pathogen.

Clinical Signs of BRD

Although there are numerous causes that contribute to BRD complex, the signs are rather consistent and easily recognizable. In a article written in 2006, Apley described the criteria for applying BRD therapy. These criteria included various combinations of respiratory rate and character, rumen fill, observed anorexia, nasal and/or ocular discharge, depression and rectal temperature (Apley 2006). In addition, dyspnea is another common clinical finding. Typical findings of histological examination of the lung tissue include bronciolitis and alveolitis with alveolar epithelial cell hyperplasia and multinucleate syncytium formation (Bryson, McFerran et al. 1979).

BRD Diagnosis

The key to successful treatment of BRD is early detection. This allows the clinician the opportunity to separate the infected cattle from the rest of the herd. Typically the earliest noticeable symptom is depression. Often depression along with an elevated rectal temperature is sufficient to tentatively diagnose a calf with BRD until laboratory tests can be performed to confirm the diagnosis (Apley 2006). It is important to realize, however, that some signs can be misleading as to the exact nature of the pulmonary involvement (rumen fill, ocular and nasal discharge, respiratory rate) or that these signs can occur late enough in the disease process that waiting for these signs to appear may decrease the chance for therapeutic success. Depression, in addition to decreased respiratory function has been shown, however, to be a good indicator of interstitial pneumonia (Apley 2006).

BRD Treatment

As mentioned above, BRD has been shown to be caused by both bacterial and viral infections. In terms of treatment of infected cattle, antimicrobial therapy has long been the focus of treatment (Apley 1997). The species of bacterial pathogens that are typically involved are *Mannheimia haemolytica*, *Histophilus somni*, and *Pasteurella multocida* (Apley 2006). In terms of viral pathogens, the focus is typically on preventative programs addressing infectious bovine rhinotracheitis (IBR), BVD, and BRSV with recent emphasis placed on testing and removal of animals that are persistently infected with BVD on arrival (Apley 2006). In terms of prevention, specific immunoprophylaxis has played an important role in the prevention and control of numerous infectious diseases. Vaccines for BHV-1, BRSV, BVDV and BPI-3 are readily available and effective (Pennathur, Haller et al. 2003; Rola, Polak et al. 2005). In terms of treatment of an infected calf, it is important to not artificially block the fever response to infection because this is one of the best defenses the animal has (Apley 2006). Ancillary treatments have been shown to be effective as well. The goal of ancillary therapy for BRD is to improve the clinical efficacy of antimicrobials. Attempting to ameliorate the harmful effects of inflammation, blocking the activity of histamine, and improving immune function are important goals of ancillary therapy. Steps to improve pulmonary function, to regulate the febrile response and to stimulate feed intake are other areas to consider. Glucocorticosteroids, NSAIDs, anti-histamines, immunomodulators such as vitamin C and levamisole, diuretics and bronchodilators have all been used successfully as ancillary treatments (Apley 1997).

BRD Impact

World wide, many studies have demonstrated a negative impact of BRD on the cattle industry. A study in 2006 showed the impact of respiratory diseases on mortality of calves to be 54-66% in Ontario, 10-61% in western Canada and 44-67% in US feedlots (Gagea, Bateman et al. 2006). Another study showed that pneumonia accounted for 52% of mortality in Ontario veal calves (taken from a pool of 4863 calves from 6 different farms) (Sargeant, Blackwell et al. 1994). Pneumonia also was shown to account for a significant portion of the mortality (proportionate mortality) in dairy calves raised on

dairy farms. Pneumonia accounted for 24% of deaths in New York's calves and 30% in Minnesota's calves (Sivula NJ 1996).

BRD Causative Agents

The viral pathogens of BRD were hinted at above and will be expanded upon in greater detail in the next few pages. In a study from 1977, serum samples were collected from early weaned calves shortly after the onset of respiratory tract disease. Seroconversion rates (a fourfold or greater rise in antibody titer) for certain viruses were obtained and were as follows: IBR – 4.3%, PI-3 – 16.3%, BVDV – 9.6%, bovine adenovirus (BAV) – 2.2% and BRSV – 45.4%. Results from this study also suggested that BRSV may facilitate infection by other viruses based on observed increases in the rate of seroconversion of the other viruses when in the presence of BRSV (Lehmkuhl and Gough 1977). In a similar study performed in 2006 (Gagea, Bateman et al. 2006), 99 calves that died or were euthanized within 60 days of their arrival at an Ontario feedlot were screened for a variety of respiratory viruses. The findings were that 35% were positive for BVDV, 9% were positive for BRSV, 6% were positive for BHV-1, 3% were positive for BPI-3 and 2% were positive for bovine corona virus (BCV) (Gagea, Bateman et al. 2006).

Bovine Viral Diarrhea (BVD)

Ever since its first description in 1946 by Olafson where it is described as “an apparently new and transmissible disease of cattle”, BVDV has been associated with a complex of clinical signs (Olafson P 1946). Over the last 60 years, a lot of progress has been made in understanding and identifying the many different clinical syndromes and diseases associated with BVDV. Currently, BVDV has been implicated in diseases affecting the reproductive, gastrointestinal, circulatory, immunologic, lymphatic, musculoskeletal, integumentary and central nervous systems as well as the respiratory tract (Brock 2004; Solis-Calderon, Segura-Correa et al. 2005). BVDV is an RNA virus in the family Flaviviridae, and is a member of the genus Pestivirus (Baker 1995; Strauss JH 2002). BVDV has a positive sense, single stranded RNA genome with one large open reading frame that encodes a polyprotein of approximately 4000 amino acids (449 Kda) (Potgieter 1995; Strauss JH 2002). Two groups of BVDV have been recognized based on genotype which are designated BVDV-I and BVDV-II. The BVDV-I group contains

viruses that are used in vaccine production, diagnostic tests, and research studies whereas BVDV-II represents isolates made from fetal calf sera, persistently infected calves born to dams vaccinated against BVDV and cattle dying from an acute form of BVDV infection termed hemorrhagic syndrome (Baker 1995). The two different species can be easily detected and differentiated using an immunoperoxidase monolayer assay (IMPA) or simultaneously with multiplex real-time PCR, a method that will be discussed later in this review (Deregt and Prins 1998; Letellier and Kerkhofs 2003; Balamurugan, Sen et al. 2006; Baxi, McRae et al. 2006). BVD has been characterized based on outbreaks of diarrhea and erosive lesions of the digestive tract. It was subsequently associated with a sporadically occurring, highly fatal, enteric disease termed mucosal disease (MD) (Baker 1995). The incubation period is approximately 5-7 days and is followed by a transient fever and leukopenia. Viremia occurs 4-5 days after infection and may persist for up to 15 days. Clinical findings include depression, anorexia, oculonasal discharge and occasionally oral lesions characterized by erosions and ulcerations, diarrhea and a decrease in milk production (Baker 1995). BVDV has the ability to induce immunosuppression that can lead to secondary bacterial or viral infections (Baker 1995). The virus has the potential to enhance disease caused by other pathogens or precipitate illness by opportunistic pathogens (Potgieter 1995). Clinical response to infection depends on numerous factors including whether the host is immunocompetent or immunotolerant to BVDV, pregnancy status, gestational age of the fetus, immune status and environmental stresses (Baker 1995). In seronegative and immunocompetent cattle, the majority of BVDV infections are subclinical. It has been estimated that 70-90% of BVDV infections occur without manifestation of clinical signs. The likely sources of BVDV for these forms of infection are cattle that are immunotolerant to and persistently infected with noncytopathic BVDV. Cattle undergoing subclinical infection may demonstrate a mild elevation in body temperature and leukopenia if closely observed (Ames 1986). There is little doubt about the importance of persistently infected (PI) cattle with respect to the epidemiology and transmission of BVDV in cattle populations. Cattle that are persistently infected with BVDV shed copious amounts of virus into their environment and are a major source of virus among newly arrived feedlot cattle. As a result, they pose a significant threat for spreading the virus and establishing acute or

primary infections in naive cattle (Campbell 2004). Therapy for BVD is supportive. Antimicrobial therapy is a consideration for treatment of secondary bacterial infections. Treatment with corticosteroids is contraindicated because they would add to the immunosuppression induced by the virus. NSAIDs are an option. Oral or intravenous fluids and electrolytes in addition to anti-diarrheals and gastrointestinal protectants are also options. Live vaccines induce broad spectrum and durable immunity but there is a concern about safety because they have been implicated in the induction of mucosal disease, immune impairment, and fetal disease. Inactivated BVDV vaccines are undoubtedly safe, but their efficacy is questionable (Baker 1995). There are a number of reasons why testing for BVDV is an important diagnostic tool. These include for the diagnosis of acute infections or reproductive failure, for detection and elimination of persistently infected animals, for testing vaccine efficacy, for quality control of biologic products, and for BVDV genotyping (Lindberg and Alenius 1999; Bhudevi and Weinstock 2001; Saliki and Dubovi 2004).

Bovine Respiratory Syncytial Virus (BRSV)

BRSV is an enveloped RNA virus with a lipoprotein coat and a negative sense ssRNA genome. It has been classified as a pneumovirus within the paramyxovirus family. Ovine RSV, Caprine RSV, Human RSV and pneumonia virus of mice are all classified in the same family paramyxoviridae (Baker and Frey 1985; Hazari, Panda et al. 2002; Strauss JH 2002; Boxus, Letellier et al. 2005). RSV was named for the characteristic merging of cells it causes, which forms multinucleated masses of protoplasm called syncytial (Baker and Frey 1985). The presence of a virus antigenically related to HRSV in cattle was predicted in the 1960's after the discovery of an inhibitor of HRSV in bovine serum that was later determined to be specific antibody (Baker and Frey 1985). Serum antibodies to RSV have been reported in cats, dogs, horses, pigs, sheep, goats, deer and antelope. BRSV wasn't isolated until the early 1970's, first in Switzerland in 1970, and then in the United States in 1974 (Baker and Frey 1985). The role of BRSV in BRD has become more and more evident as diagnostic techniques have been refined over the years. Through improvements in virus isolation, the use of immunofluorescent tests, and better recognition of clinical signs and pathology associated with BRSV infections, its impact on BRD is becoming more apparent (Castleman,

Torres-Medina et al. 1985; Achenbach, Topliff et al. 2004). The incidence of BRSV-associated disease in the cattle population is not known, but based on the studies of antibody prevalence, infection with BRSV appears to be a common event. Studies have shown that antibody prevalence in Iowa, Alabama, Oklahoma and Minnesota are 81%, 67%, 73.6% and 65.5%, respectively (Baker and Frey 1985). Outbreaks of BRSV can reach epidemic proportions as was seen in Japan when an outbreak affected more than 40,000 cattle (Baker and Frey 1985). While the exact mechanism of transmission is unknown, it appears that aerosol transmission can occur and that the route of entry is either the respiratory tract or by ingestion of the virus. The onset of clinical signs is sudden and the virus appears to have a very short incubation period. Unlike BVD, it is unclear if a chronic carrier state can occur in cattle, although it appears that cattle are the principle reservoirs of infection (Baker and Frey 1985). While it is not known if sheep or goat RSV can cause disease in cattle, sheep have been experimentally infected with BRSV (Al-Darraj, Cutlip et al. 1982). Typical for respiratory diseases of cattle, RSV infections tend to peak during the fall and winter seasons but they have been reported during other times of the year as well. In terms of susceptibility, there appears to be no difference between female and male cattle, but there appears to be differences based on breed with red beef breeds reported to be more susceptible (Baker and Frey 1985). Some early clinical signs of infection are decreased feed consumption, mild depression, mucoid nasal discharge, salivation, lacrimal discharge, increased rate of respiration, and rectal temperatures ranging from 40-42.2°C. As the disease progresses, dyspnea becomes more pronounced with open mouth breathing, frothing of saliva, and formation of subcutaneous edema around the throat and neck (Baker and Frey 1985; Castleman, Torres-Medina et al. 1985). Auscultatory findings include increased bronchial and bronchovesicular sounds with fine crackles, which are consistent with emphysema (Baker and Frey 1985). Scanning electron microscopy indicates that the ciliated respiratory epithelium is almost completely destroyed in susceptible calves 8-10 days after experimental infection with BRSV, which would indicate that pulmonary clearance may also be compromised by BRSV allowing secondary pulmonary infections (Eis 1979). Antibiotic therapy is indicated for treatment because of the common occurrence of bacterial pneumonia secondary to BRSV. Sensitivity testing should be performed when

possible to assure effective treatment (Baker and Frey 1985). Corticosteroids and anti-histamines have been used successfully in severe cases of BRSV, but should not be used indiscriminately in cases of BRD (Bohlender RE 1982). A vaccine against BRSV has been available since 1978, which has been proven effective as a preventative measure against BRSV infection (Kucera CJ 1983).

Bovine Parainfluenza Virus – 3 (BPI-3)

Unlike BRSV and BVDV, very little is known about the impact of BPI-3 on BRD. It is rarely screened for in diagnostic labs and literature searches reveal very little information as well, making it difficult to assess what influence it has on this disease complex. A study performed in 1966 found that experimental infection of calves with PI-3 produced clinical signs similar to BRD, including pneumonic lesions but of less severity than those witnessed with a concurrent infection (Omar, Jennings et al. 1966). A study in 1994 suggested that PI-3 makes the body more susceptible to secondary bacterial infections by inhibiting bactericidal functions of phagocytic cells (Dyer, Majumdar et al. 1994). PI-3 is in the genus *Respirovirus* and the family *Paramyxoviridae*. It has a negative ssRNA structure for its genome and is closely related to HPI-3 (Strauss JH 2002). The first clinical signs seem to be depression, reduced appetite and coughing, which progresses into respiratory distress and dyspnea along with nasal discharge (Bryson, McFerran et al. 1979). BPI-3 was first isolated in the USA from the nasal mucus of cattle showing clinical signs of shipping fever. Its distribution in cattle has been shown to be worldwide. Most reports of BPI-3 virus activity have been in groups of young cattle with respiratory diseases such as enzootic calf pneumonia and shipping fever. Auscultation of severely infected cattle often reveals harsh lung sounds accompanied by a marked increase in respiratory rate. Emphysematous crackling can often be detected (Bryson, McFerran et al. 1979). Live, attenuated vaccines are currently available for BPI-3 (Pennathur, Haller et al. 2003; Greenberg, Walker et al. 2005).

Laboratory Diagnostic Methods

Numerous methods have been used over the years for detection and differentiation of the various pathogens that contribute to the causation of the BRD complex. A few of these will be discussed below. Of the many options available, the two that are the most prominent are enzyme linked-immunosorbant assay (ELISA) and

polymerase chain reaction (PCR) (West, Bogdan et al. 1998; Saliki and Dubovi 2004). When considering a diagnostic test, the most important considerations are rapidity, ease of use, specificity and sensitivity. Rapid detection of viral infections and understanding of viral pathogenesis are crucial for the prevention of infectious disease outbreaks, for the development of antiviral drugs and, for biodefense. Earlier detection of BRD would enable a more targeted treatment regime and earlier isolation of infected individuals. Some methods that have proven useful for the detection of BRD pathogens are enzyme immunoassays (EIA) (Gogorza, Moran et al. 2006), monoclonal antibody-based immunoperoxidase monolayer assays (Deregt and Prins 1998), and infrared thermography, which allows for earlier detection of BRD (up to 4-6 days prior to the development of clinical signs) (Schaefer, Cook et al. 2007). Molecular beacons have also been used to detect the viral genome and to characterize a clinical isolate of BRSV in living cells. Molecular beacons are dual-labeled, hairpin oligonucleotide probes with a reporter fluorophore at one end and a quencher at the other (Santangelo, Nitin et al. 2006). ELISA and PCR will be discussed in more detail below.

ELISA

ELISA has been shown to be an invaluable test for determining the presence of BVD (Gogorza, Moran et al. 2005; Hill, Reichel et al. 2007) and BRSV (Graham, Foster et al. 1999; Hazari, Panda et al. 2002) in clinical samples. ELISA has been shown to be highly sensitive, specific, rapid, and possesses the advantages of simplicity and adaptability to automation (Hazari, Panda et al. 2002). Alternative methods such as virus isolation and immunohistochemistry are time-consuming and laborious and results may be difficult to interpret whereas ELISA technology lends itself to large-scale testing of a greater number of samples (Hill, Reichel et al. 2007) but is unable to test for multiple pathogens simultaneously..

Reverse Transcriptase PCR (RT-PCR)

PCR has been used as the new gold standard for detecting a wide variety of templates across a range of scientific specialties, including virology (Mackay, Arden et al. 2002). Existing combinations of PCR and detection assays have been used to obtain quantitative data with promising results. However, these approaches have suffered from the laborious post-PCR handling steps required to evaluate the amplicon (Mackay, Arden

et al. 2002). Traditional detection of amplified DNA relies on electrophoresis of the nucleic acids in the presence of ethidium bromide and visual or densitometric analysis of the resulting bands after irradiation by ultraviolet light. Southern blot detection of amplicon using hybridization with labeled oligonucleotide probes is also time consuming and requires multiple PCR product handling steps, further risking a spread of amplicon throughout the laboratory (Mackay, Arden et al. 2002). PCR-ELISA may be used to capture amplicon onto a solid phase using biotin or digoxigenin-labeled primers, oligonucleotide probes or directly after incorporation of digoxigenin into the amplicon. Once captured, the amplicon can be detected using enzyme-labeled avidin or anti-digoxigenin reporter molecule similar to those used in the standard ELISA format (Mackay, Arden et al. 2002). RT-PCR has been used successfully to detect BVDV (Bhudevi and Weinstock 2001; Deregt, Carman et al. 2002; Mahlum, Haugerud et al. 2002) and BRSV (Valarcher, Bourhy et al. 1999; Almeida, Domingues et al. 2004) in experimental and clinical samples.

Real-Time PCR

In contrast to conventional RT-PCR, Real-Time RT-PCR allows one to visualize the amplification of the amplicon as it progresses in real time. This approach has provided great insight into the kinetics of the reaction. The monitoring of accumulating amplicon in real time has been made possible by the labeling of primers, probes or amplicon with fluorogenic molecules. Real-time has gained wider acceptance because of its improved rapidity, sensitivity, reproducibility, and the reduced risk of carry-over contamination (Mackay, Arden et al. 2002). Increased speed is largely the result of reduced cycle times, removal of post-PCR detection procedures, and the use of fluorogenic labels and sensitive methods for detecting their emissions. Disadvantages of using real time in comparison to conventional PCR include the inability to monitor amplicon size without opening the system, the incompatibility of some platforms with some fluorogenic chemistries, and the relatively restricted multiplex capabilities of current applications. In addition, start up cost is high and may be prohibitive to low through put laboratories (Mackay, Arden et al. 2002). Real-Time PCR has been used successfully to detect BVDV (Letellier and Kerkhofs 2003; Young, Thomas et al. 2006) and BRSV

(Achenbach, Topliff et al. 2004; Boxus, Letellier et al. 2005) in experimental and clinical samples.

Multiplex Real-Time PCR

Multiplexing is the use of multiple primers to allow amplification of multiple templates within a single reaction. The term multiplex real-time PCR is more commonly used to describe the use of multiple fluorogenic oligoprobes for the discrimination of multiple amplicons (Mackay, Arden et al. 2002). As the number of fluorophores has increased, this method has become much more common because of its ability to differentiate multiple amplicons in a single reaction. So far it has been used for the detection and typing of different BVDV strains grown on Madin-Darby bovine kidney (MDBK) cells (Baxi, McRae et al. 2006) and in clinical samples (Balamurugan, Sen et al. 2006).

Materials and Methods

All Procedures were conducted in Dr. Pastey's labs located in Rooms No. 301, 308 and 309 Dryden Hall and the Veterinary Diagnostic Lab on OSU Campus.

Viruses and cell cultures

BVD viral reference strain New York-1 was obtained from National Animal Disease Center, Ames, IA. BRSV strain A51908 (ATCC VR-794) and BPIV-3 strain SF-4, (ATCC VR-281) were obtained previously from the American Type Culture Collection. Vero and Madin Darby bovine kidney (MDBK) cells were maintained in minimal essential medium supplemented with 10% horse serum, L-glutamine, and antibiotics.

Primer and probe design for multiplex real-time PCR

Primer and Taqman probe sequences were selected from an alignment of nucleotide sequences of the BRSV, BVD and BPIV3 from GenBank (Table 1). The alignment was performed to select a highly conserved region for each virus. The PCR primers and probes were optimized with Primer Designer version 2.0, to perform with an annealing temperature of 55°C. Additionally, the program checked interactions between primers and probes. Two different probes were used for both BRSV and BVD. All primers and probes (Table 1) were purchased from Sigma Genosys.

Table 1

Sequences of primers and probes for multiplex real-time PCR

<u>Primer/probe</u>	<u>Position</u>	<u>Composition</u>
<u>BRSV</u>		
Forward Primer	364-388	5'-GTCAGCTTAACATCAGAAGTTCAAG-3'
Probe	434-468	5'-FAM-AAGAGATGGGAGAGGTAGCTCCAGAATACAGACAT-BHQ-1-3'
Probe	434-468	5'-TET-AAGAGATGGGAGAGGTAGCTCCAGAATACAGACAT-BHQ-1-3'
Reverse Primer	477-501	5'-ACATAGCACTATCATACCACAATCA-3'
<u>BPIV3</u>		
Forward Primer	507-532	5'-GGGAGTGATCTTGAGTATGATCAAGA-3'
Probe	558-593	5'-TET-ACTTCTACAATCGAGGATCTTGTTTCATACTTTTGGGA-Dabcyl-3'
Reverse primer	603-625	5'-TGGATTATAAGGGCTCCAAGACA-3'
<u>BVD</u>		
Forward Primer	5'UTR	5'-GGGNAGTCGTCARTGGTTCG-3'
Probe	5'UTR	5'-Texas Red-CCAYGTGGACGAGGGCAYGC-TAMRA-3'
Probe	5'UTR	5'-FAM-CCAYGTGGACGAGGGCAYGC-BHQ-1-3'
Reverse Primer	5'UTR	5'-GTGCCATGTACAGCAGAGWTTTT-3'

Detection and differentiation of pathogens in cell culture

Virus stocks: BRSV and BVDV were cultured on MDBK cells, and BPIV3 was cultured on Vero cells. About 5 ml of each virus was prepared, and the stocks were stored in 0.5-ml aliquots at -80°C. The limit of sensitivity of the multiplex real-time RNA PCR was determined by testing 10-fold dilutions of the 50% tissue culture infectious dose (TCID₅₀) for each virus type.

RNA extraction: Ambion MagMAX magnetic bead system (Ambion) was used for RNA extraction in the development of the assay. All samples were extracted according to the manufacturer's instructions and stored at -80°C. Specifically, 50 µL of the prepared viral sample was added to 130 µL of the lysis/binding solution and mixed. Then, 20 µL of the magnetic bead mix was added and the solution was shaken for 10 minutes to fully lyse the viruses and bind the RNA. The sample was then placed on a magnetic stand and the supernatant was extracted. The beads were washed twice with 150 µL of wash solution-1 and then twice with 150 µL of wash solution-2. After each wash step, the supernatant was extracted in the same manner as above. Finally, 35 µL of elution buffer was added to each well in order to separate and extract the RNA from the magnetic beads and the

supernatant was collected. The absorbance of the sample at 260nm was measured to quantify the captured RNA and tested by multiplex real-time PCR under the conditions described below.

Real-time PCR conditions: The assays were optimized first in a monospecific real-time PCR; subsequently, multiplex reactions were performed for the following samples: BRSV/BPI3, BRSV/BVD, BPI3/BVD and BRSV/BPI3/BVD. The kit used for this assay was the SuperScript III Platinum One-Step Quantitative RT-PCR kit from Invitrogen. Real-time PCR was performed in 25 μ L of reaction mixture consisting of 12.5 μ L of 2X Reaction mix (Invitrogen Superscript III Platinum One-Step Quantitative RT-PCR Mix), 5mM MgSO₄, 0.5 μ L SuperScript III RT/Platinum *Taq* mix, 0.5 μ L of RnaseOUT (Invitrogen), 0.4 μ M concentrations of each primer, 0.2 μ M concentrations of Taqman Probe and 5 μ L of template. The PCR thermal profile consisted of an initial cDNA step of 30 min at 48°C followed by 2 min at 95°C and 45 cycles of 15 s at 95°C, 30 s at 55°C, and 30 s at 72°C. Amplification, detection, and data analysis was performed with the iCycler IQ real-time detection system (Bio-Rad).

Agarose gel electrophoresis: A 2% agarose gel (500mg agarose, 25mL 1% TAE buffer and 2 μ L of ethidium bromide) was used to confirm amplification by PCR. A low range ladder (from Fermentas Life Sciences) was used as a reference and every other well contained 1 μ L of loading dye and 5 μ L of sample.

Equipment and Location: In this study, the iCycler IQ and the Opticon real-time PCR detection platforms, located in 301 Dryden Hall and the Veterinary Diagnostic Lab at Oregon State University, respectively, were used. The iCycler can detect four different fluorophores in a single well and the Opticon can detect two. All the Procedures were conducted in rooms 308, 309, & 301 Dryden Hall, Dept. of Biomedical Science and the Oregon State University Veterinary Diagnostic Laboratory.

Detection and differentiation of pathogens in clinical samples

Clinical samples: From December 2004 to December 2005, a number of respiratory samples (nasal swabs, nasopharyngeal aspirates, and bronchoalveolar lavage samples) and serum samples were received by the Veterinary Diagnostic Laboratory for routine culture and diagnosis of viral infection. From each sample, an aliquot was stored at -70°C for PCR analysis.

Results

RNA Isolation

As was mentioned in the methods section, the virus samples were isolated using the Ambion MagMAX magnetic bead system. This system was used for RNA isolation from both cultured cells and from clinical samples collected over the course of one year (also referenced in the methods section.) The extracted product's RNA concentration was then tested by measuring the absorbance of the sample at a wavelength of 260nm. The typical absorbance varied from 0.047 to 1.039 which correlates to RNA concentrations of 37.60mg/ μ L to 332.5 μ g/mL.

Real-Time Monoplex PCR

The first assays performed were monoplex reactions using cultured virus stock, as described above. Calibration curves were determined by diluting the purified samples in tenfold dilutions from 10^{-1} to 10^{-7} . This data was then used to determine the efficiency of the reactions.

A number of modifications to the protocol were made in an attempt to optimize results of the PCR assay. For the monoplex reactions, the only modification that was necessary was adjusting the concentration of MgSO_4 in the master mix. The stock concentration was 3mM and the concentrations that seemed to be the most effective were between 4 and 6mM. Any concentration above 6mM seemed to negatively impact the outcome of the assay. For the data below, the graphs with grids were performed on the Biorad iCycler and the graphs without a grid were performed on the Biorad Opticon. On the graphs, the x-axis represents the cycle number. The $c(t)$ value is the threshold cycle, which is the cycle number at which the fluorescence generated within the reaction crosses the threshold. This value indicates when a sufficient number of amplicons have accumulated. In the graphs of the calibration curves, the samples with the least dilution have the lowest $c(t)$ and the $c(t)$ values increase as the dilution increases. The y-axis represents the fluorescence detected. When the probe binds to the target, it fluoresces and, therefore, the greater the fluorescence detected, the greater the quantity of amplicon. The amplicon is the amplified sequence of DNA in the PCR process.

Real-Time Monoplex PCR on control samples

Below are the calibration curves for the three viruses studied at different concentrations of MgSO_4 . The concentration of MgSO_4 seemed to greatly impact the efficacy of the assay.

BRSV 3mM MgSO_4

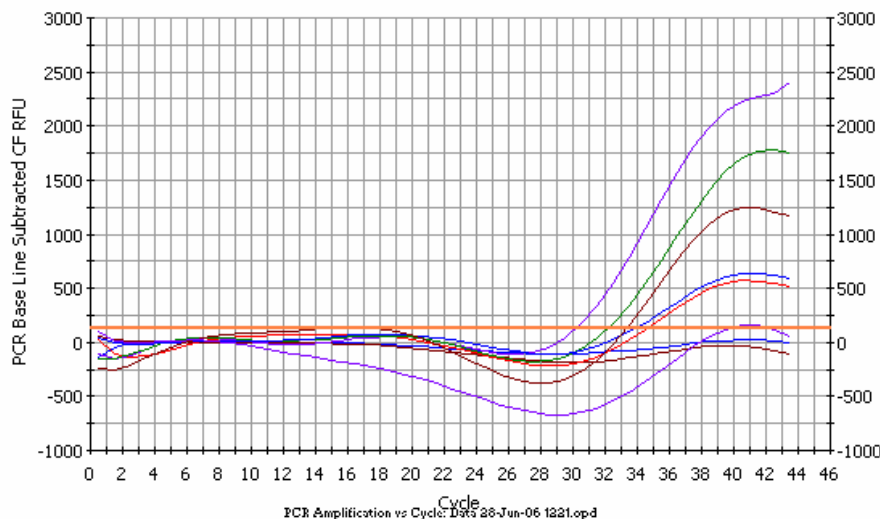


Figure 1.1: In the graph above, the least dilute sample is purple (lowest $c(t)$ value), the most dilute sample is blue, and the negative control is brown.

BRSV 4mM MgSO_4

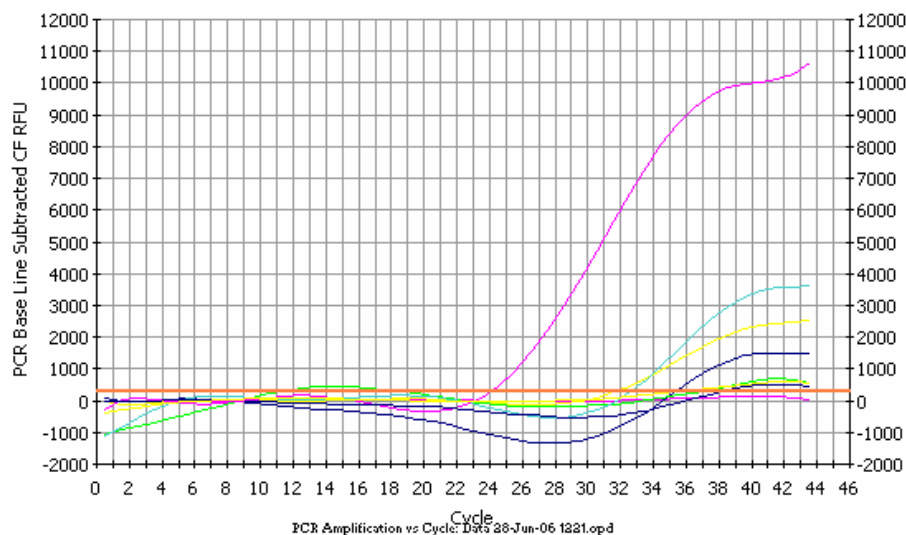


Figure 1.2: In the graph above, the least dilute sample is pink (lowest $c(t)$ value), the most dilute sample is blue, and the negative control is purple.

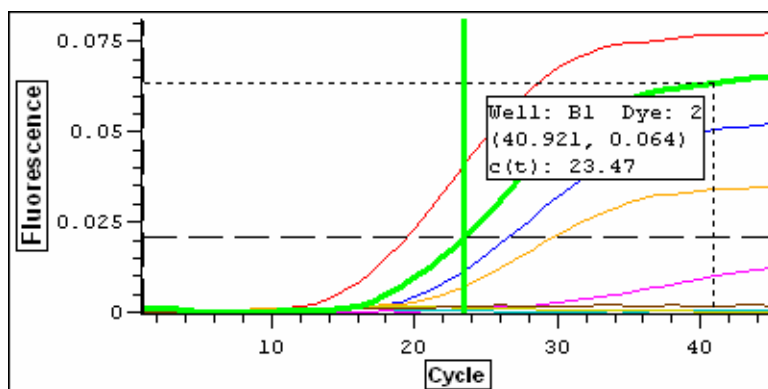
BRSV 5mM MgSO₄

Figure 1.3: In the graph above, the least dilute sample is red (lowest $c(t)$ value), the most dilute sample is pink, and the negative control is brown.

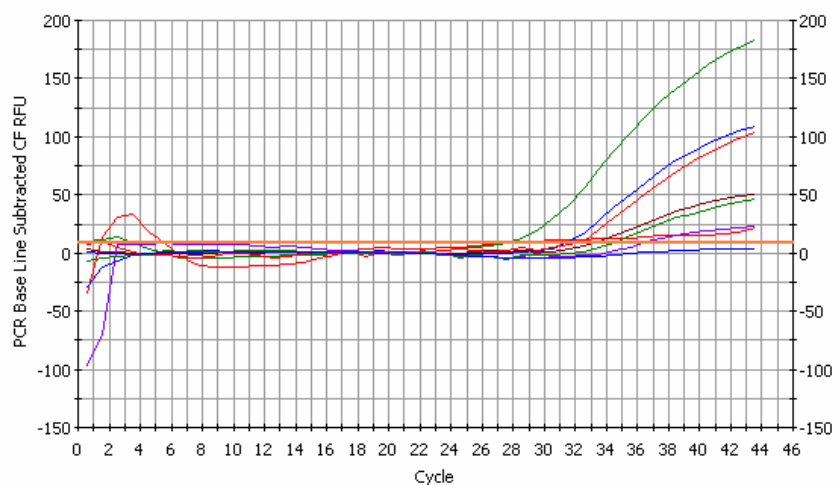
BRSV 6mM MgSO₄

Figure 1.4: In the graph above, the least dilute sample is green (lowest $c(t)$ value) the most dilute sample is blue, and the negative control is also blue.

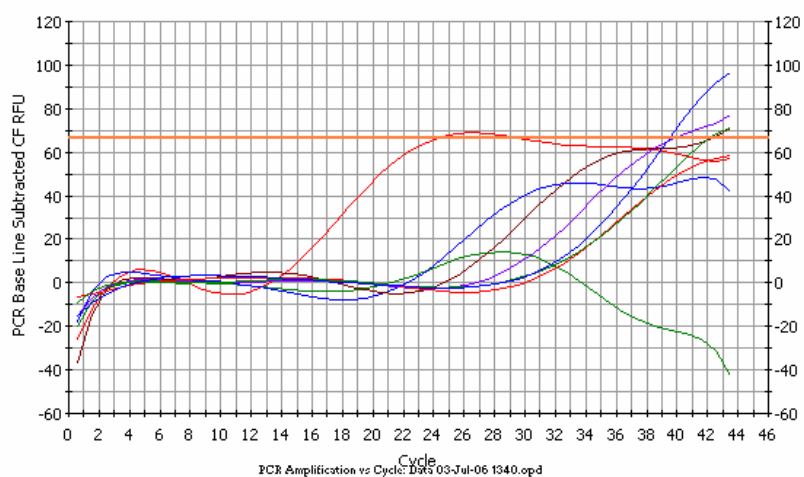
BPI3 3mM MgSO₄

Figure 2.1: In the graph above, the least dilute sample is red (lowest $c(t)$ value) the most dilute sample is green, and the negative control is also green.

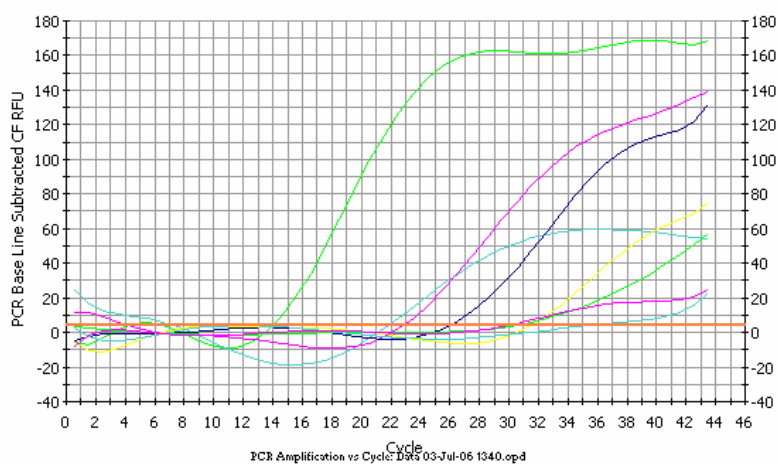
BPI3 4mM MgSO₄

Figure 2.2: In the graph above, the least dilute sample is green (lowest $c(t)$ value) the most dilute sample is pink, and the negative control is light blue.

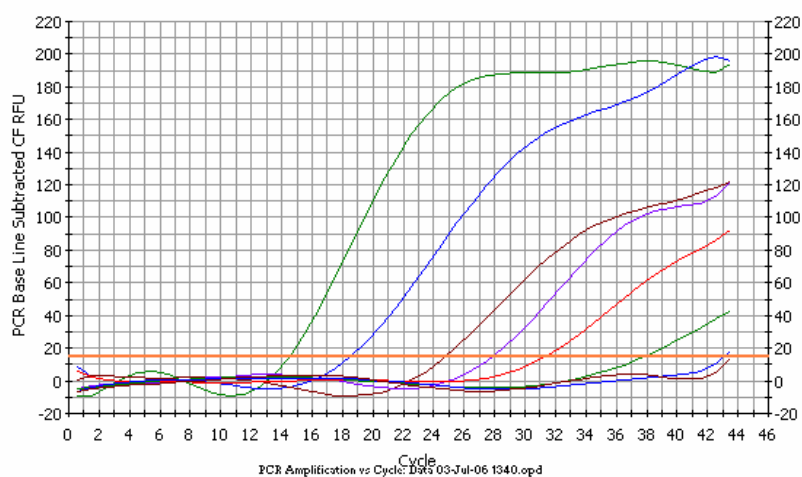
BPI3 5mM MgSO₄

Figure 2.3: In the graph above, the least dilute sample is green (lowest $c(t)$ value) the most dilute sample is blue, and the negative control is brown.

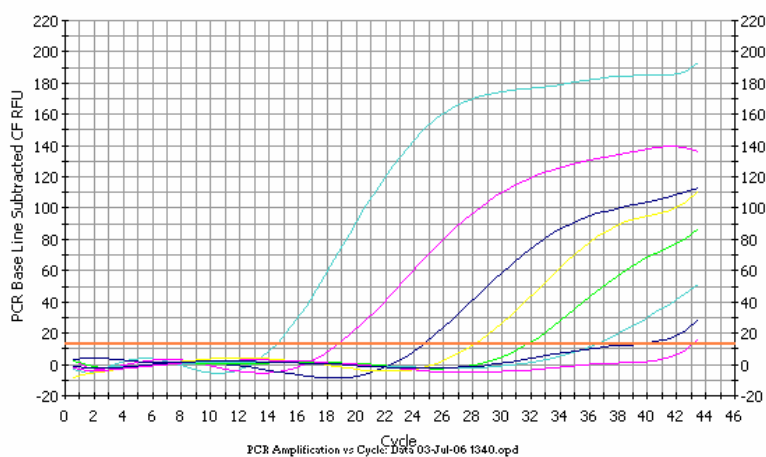
BPI3 6mM MgSO₄

Figure 2.4: In the graph above, the least dilute sample is light blue (lowest $c(t)$ value) the most dilute sample is blue, and the negative control is pink.

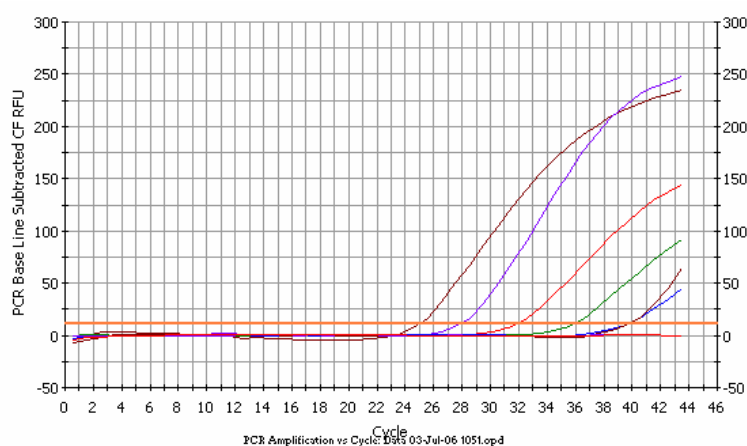
BVD 3mM MgSO₄

Figure 3.1: In the graph above, the least dilute sample is brown (lowest $c(t)$ value) the most dilute sample is blue, and the negative control is red.

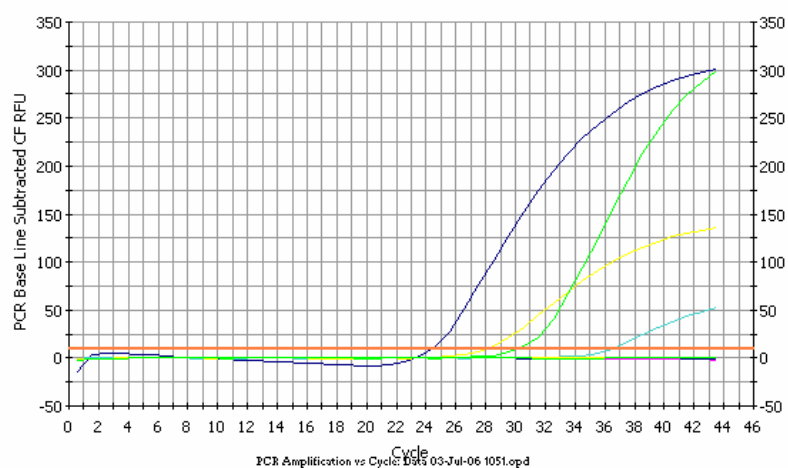
BVD 4mM MgSO₄

Figure 3.2: In the graph above, the least dilute sample is blue (lowest $c(t)$ value) the most dilute sample is red, and the negative control is brown.

BVD 5mM MgSO₄

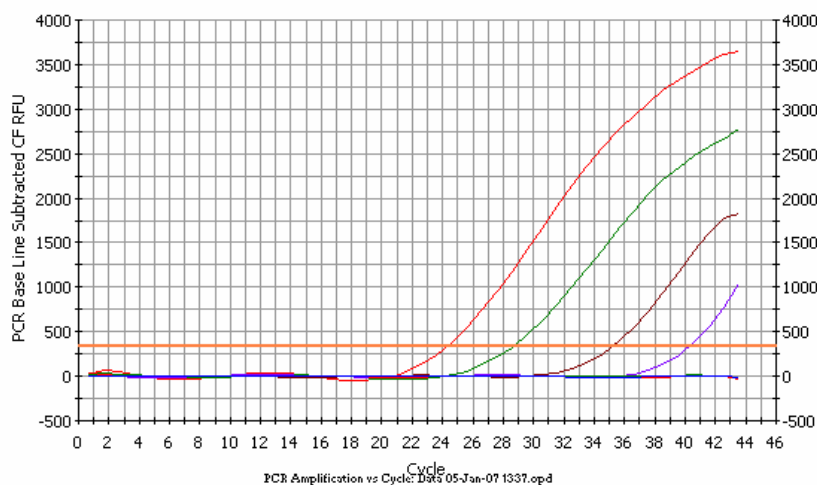


Figure 3.3: In the graph above, the least dilute sample is red (lowest c(t) value) the most dilute sample is blue, and the negative control is yellow.

BVD 6mM MgSO₄

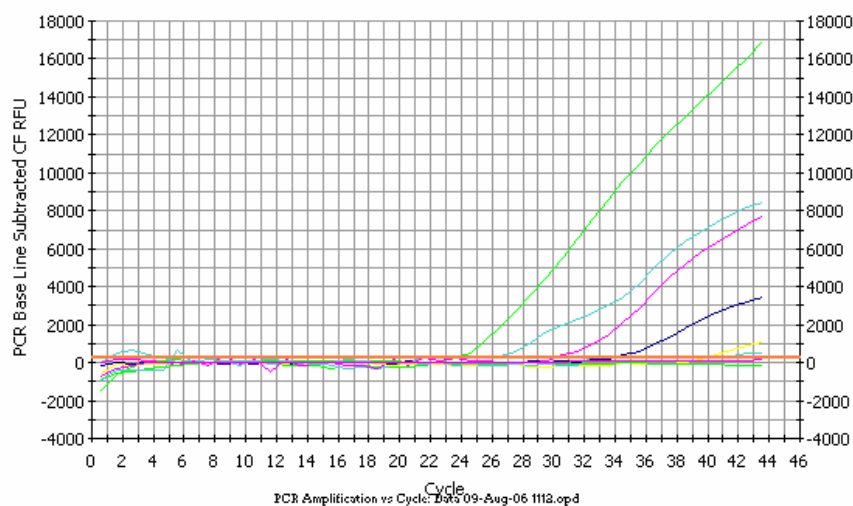


Figure 3.4: In the graph above, the least dilute sample is green (lowest c(t) value) the most dilute sample is yellow, and the negative control is brown.

Efficiency of the Reactions

The efficiency of the reactions were determined graphically using Microsoft excel. The efficiencies of all three reactions were calculated for a concentration of MgSO₄ of 5mM. The log of the sample concentrations versus the c(t) values were graphed as shown below.

Table 2: $c(t)$ values of monoplex PCR assays for each virus (from calibration curves with $MgSO_4$ concentrations of 5mM.)

Dilution	log (dilution)	BRSV	BPI3V	BVDV
0.1	-1	19.45	15	24.3
0.01	-2	23.47	19.3	28.6
0.001	-3	26.57	25.7	
0.0001	-4	29.81	28.8	35.2
0.00001	-5		32.5	40.2
0.000001	-6		39.4	
0.0000001	-7			

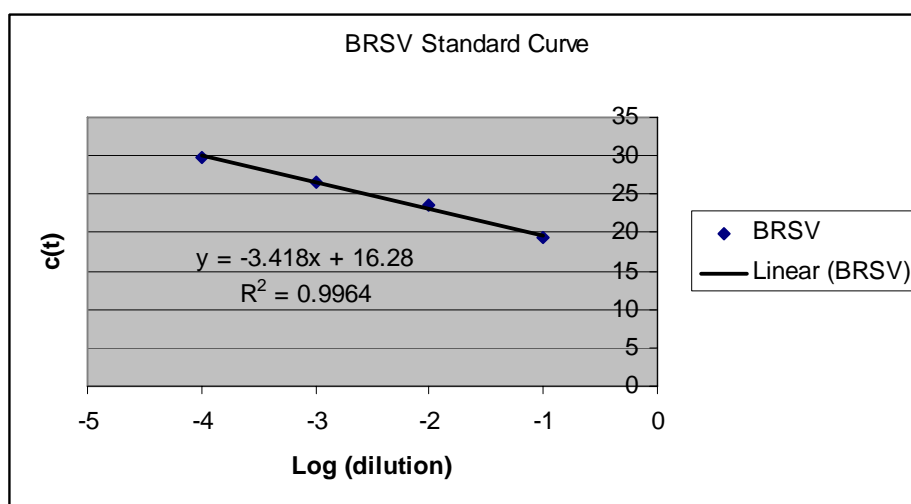


Figure 4.1: Graph of data from Table 2 for BRSV.

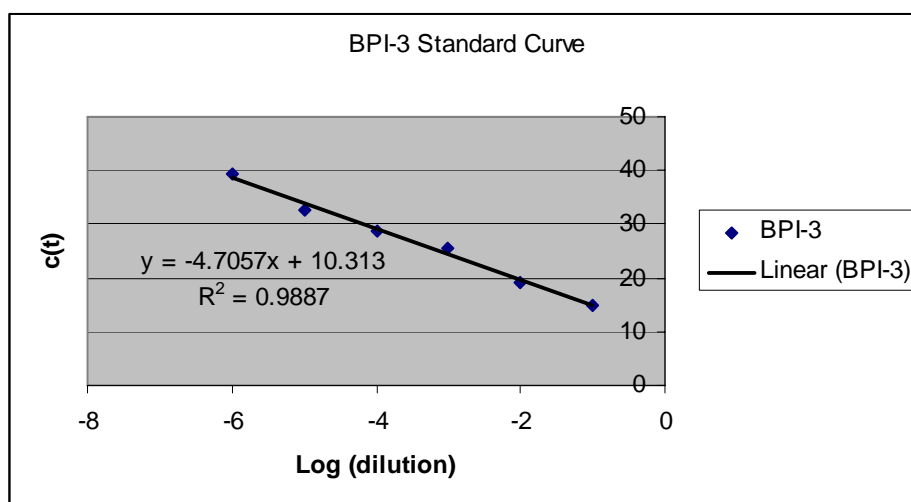


Figure 4.2: Graph of data from Table 2 for BPI3V.

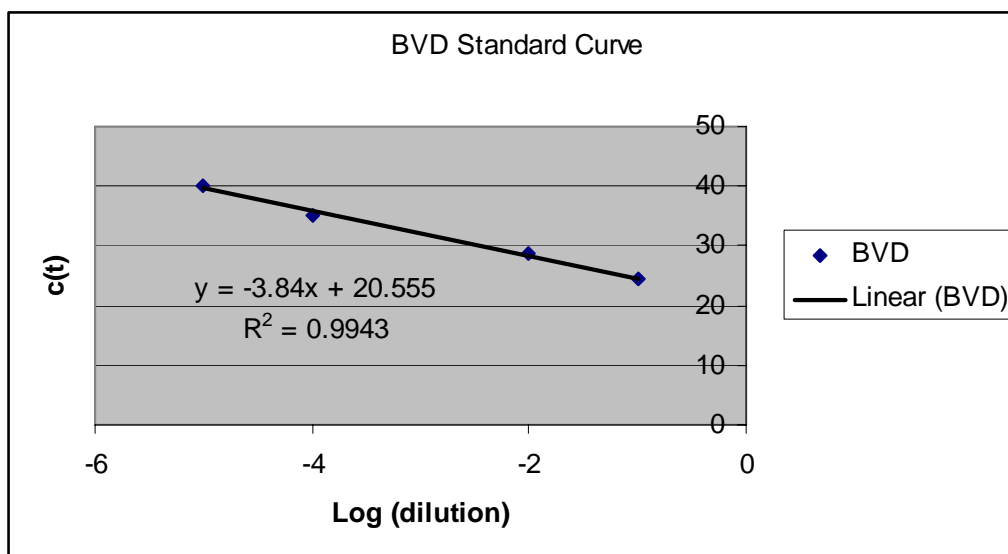


Figure 4.3: Graph of data from Table 2 for BVDV.

The efficiency of the reaction was calculated by the following equation:

$E = 10^{(-1/\text{slope})} - 1$. The efficiency of PCR should be 90-100% meaning doubling of the amplicon at each cycle. This corresponds to a slope of -3.1 to -3.6 in the $C_{(t)}$ vs log-template standard curve.

Table 3: Efficiencies of the PCR assay for each virus.

Virus	Slope	Efficiency	R Squared
BRSV	-3.418	0.96	0.9964
BPI3	-4.7057	0.63	0.9887
BVD	-3.84	0.82	0.9943

The efficiencies of the monoplex reactions for BRSV, BPI3V and BVDV were 96%, 63% and 82%, respectively.

Agarose Gel Electrophoresis for real-time monoplex PCR products:

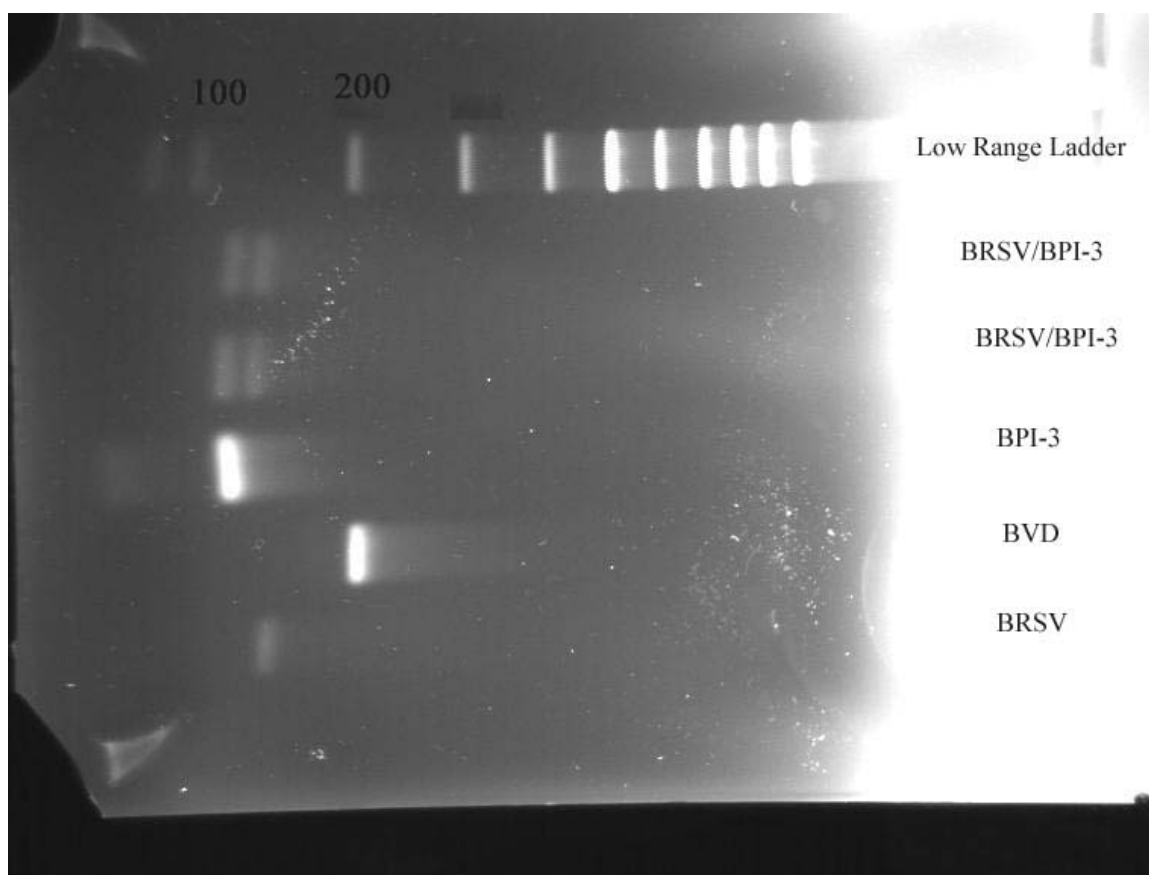


Figure 5: Agarose gel of monoplex and multiplex assay products. Wells from top: 1) low-range ladder, 2) BPI3V/BRSV duplex, 3) BPI3V/BRSV duplex, 4) BPI3V, 5) BVDV and 6) BRSV.

Agarose gels were used to verify amplification. On the gel above, a distinct band for BRSV, BPI3V and BVDV can be clearly seen as well as 2 bands in the columns that were run on a multiplex reaction samples.

Real-Time monoplex PCR on Clinical samples:

Monoplex assays were run on 100 clinical samples screening for BRSV, BPI3V and BVDV separately. The results are shown below.

Figure 6: BPI3V monoplex clinical samples

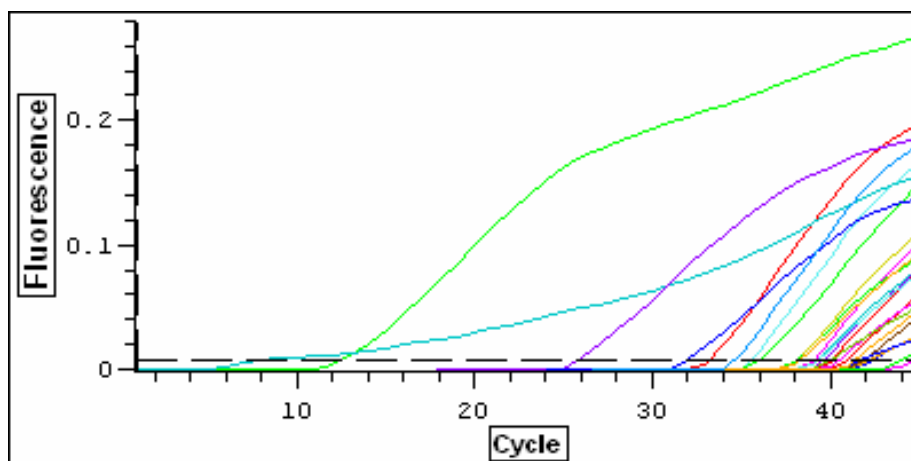


Figure 7: BRSV monoplex clinical samples

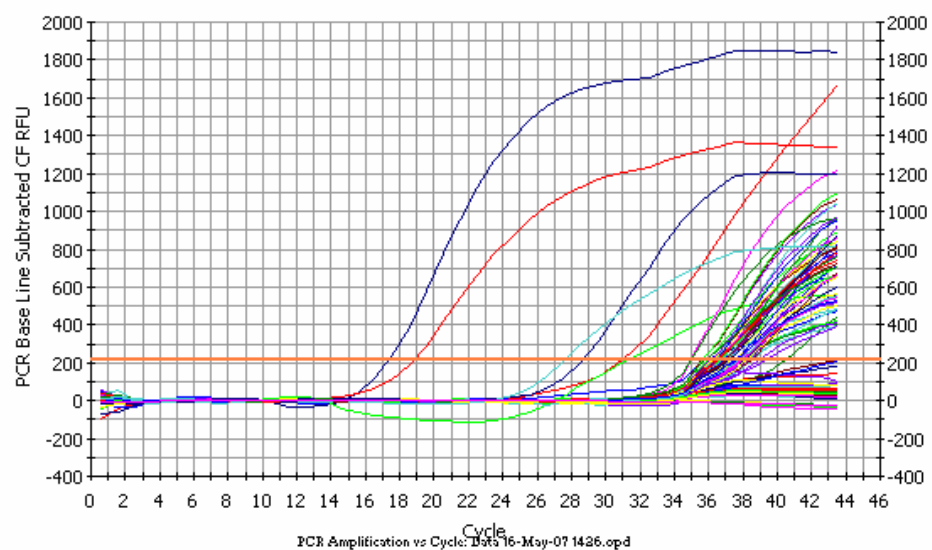
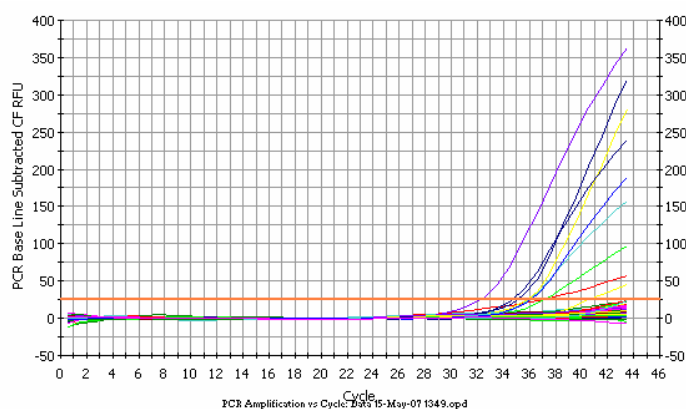


Figure 8: BVDV monoplex clinical samples



There appeared to be a lot of false positives that occurred late in the run with $c(t)$ values greater than 36. Thus, these were not counted as positive results. The data from the monoplex reactions will be included in a table at the end of the results section along with the multiplex data and will be discussed in the discussion section.

Real-Time Multiplex PCR on control samples:

Numerous attempts were made to perfect the multiplex reaction. The various combinations attempted were BRSV/BPI3V, BRSV/BVDV, BPI3V/BVDV and BRSV/BPI3V/BVDV. The results of these assays were mixed and in the process of doing these experiments, a number of modifications to the protocol were attempted. These included decreasing the primer and probe concentrations, modifying the annealing temperature and length of time, different sources of water, and preparing the run in a sterile environment. The effect of each of these modifications will be discussed in detail in the discussion section. When a multiplex reaction is shown graphically, the virus in bold face is the virus whose fluorophore is being detected for that specific graph.

The first attempt at a multiplex reaction was BPI3V/BVDV using the same protocol as was used for the monoplex reactions. Once again, the calibration curves below show the general trend of the sample with the lowest $c(t)$ value being the sample with the most concentrated amount of RNA.

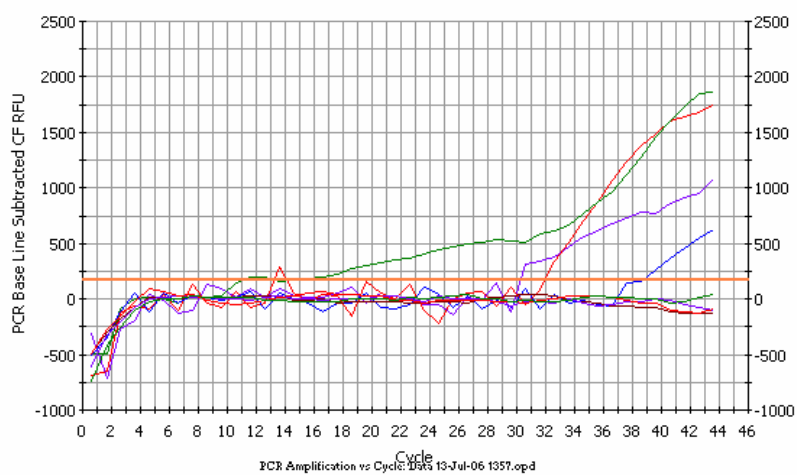
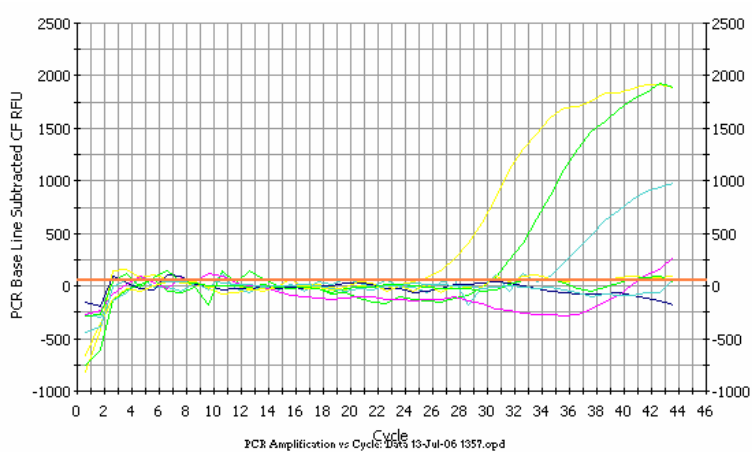
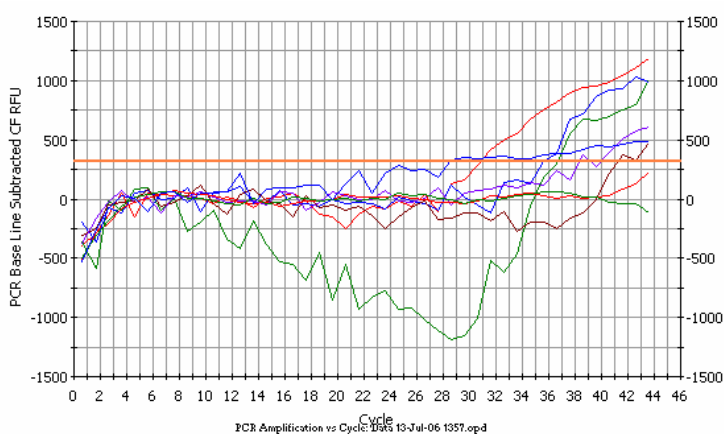
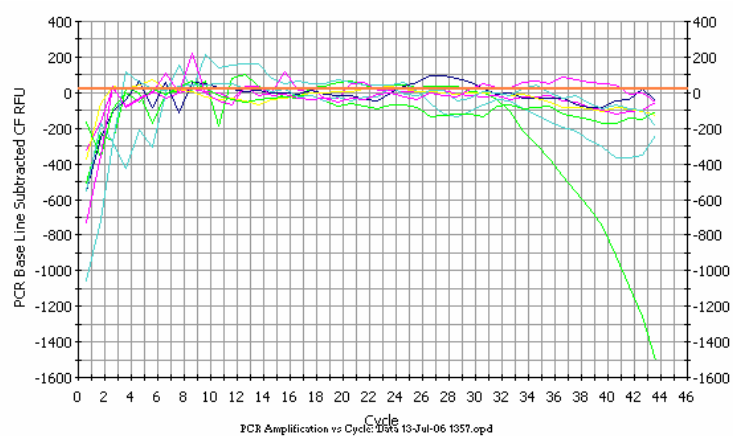
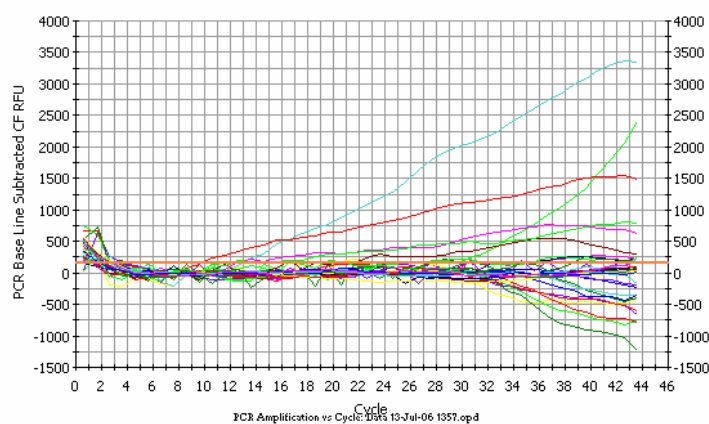
Figure 9.1: BPI3V/BVDV 3mM MgSO₄Figure 9.2: BPI3V/BVDV 4mM MgSO₄Figure 9.3: BPI3V/BVDV 5mM MgSO₄

Figure 9.4: BPI3V/BVDV 6mM MgSO₄Figure 9.5: BPI3V/BVDV (3mM – 6mM MgSO₄ combined on one graph)

The duplex reaction of BRSV/BPI3V was then attempted with similar results.

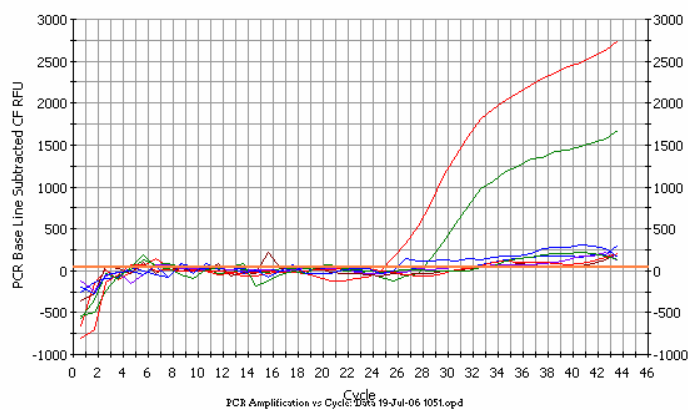
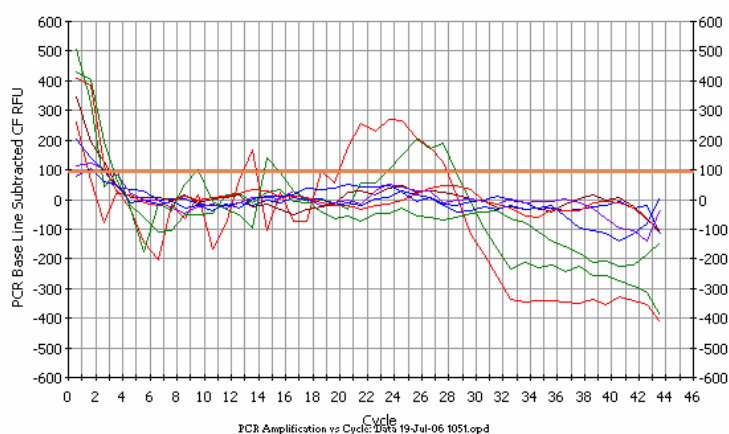
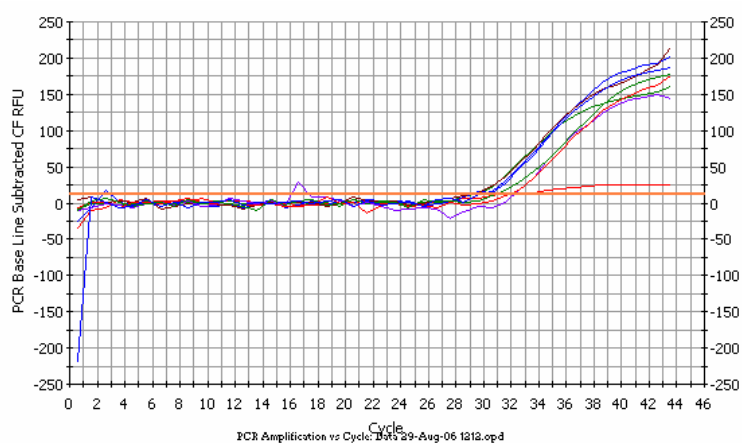
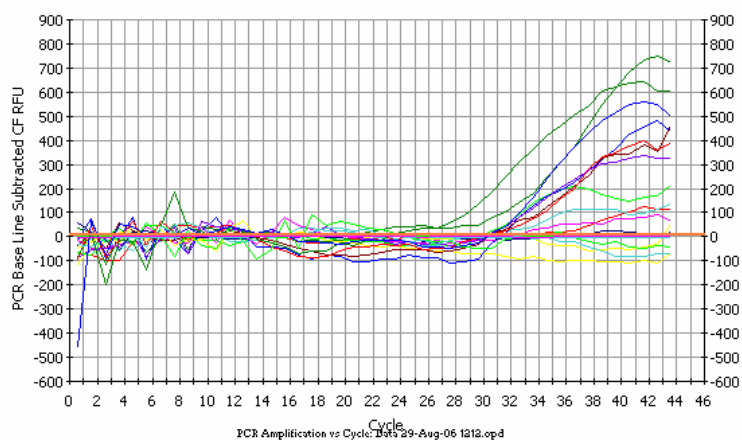
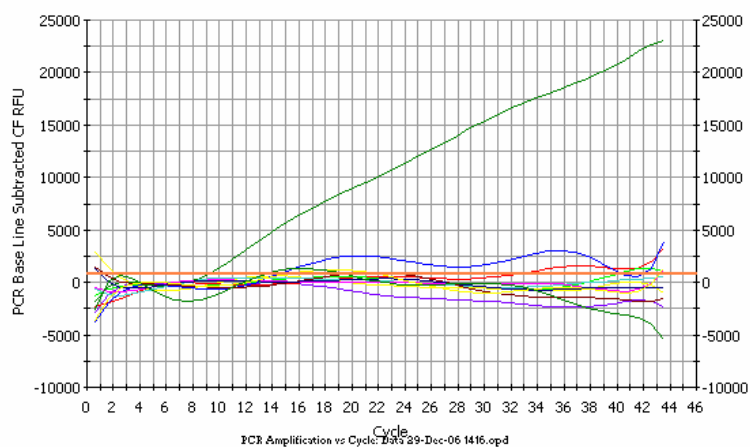
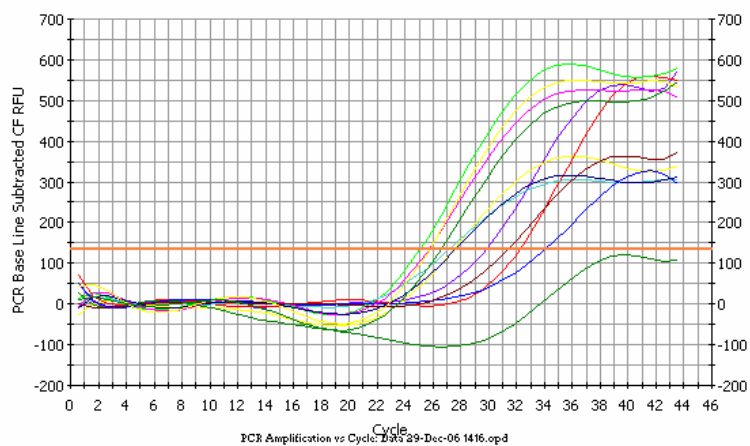
Figure 10.1: BRSV/BPI3V 5mM MgSO₄

Figure 10.2: BRSV/BPI3V 5mM MgSO₄

The third attempt was BRSV/BVDV in order to complete the available options for duplex reactions and get an idea of which virus combinations would cause the most problems in a multiplex reaction of all three.

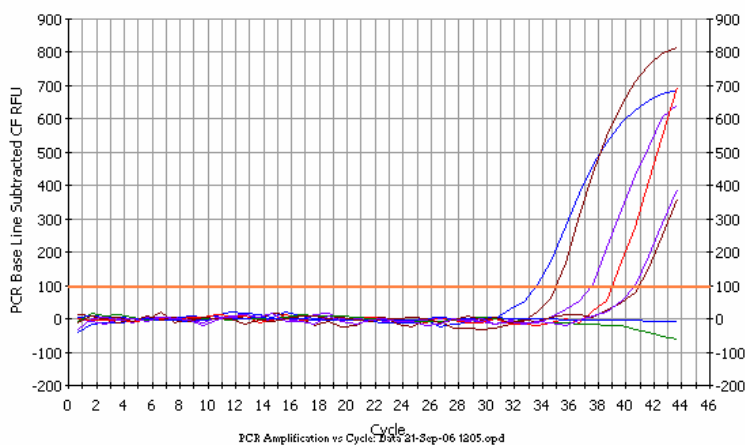
Figure 11.1: BRSV/BVDV 4mM MgSO₄

This graph is a probably the result of primer interference causing every dilution to come up with the same c(t) value.

Figure 11.2: **BVDV/BRSV** 4mM MgSO₄Figure 11.3: **BRSV/BVDV** 5mM MgSO₄Figure 11.4: **BRSV/BVDV** 5mM MgSO₄

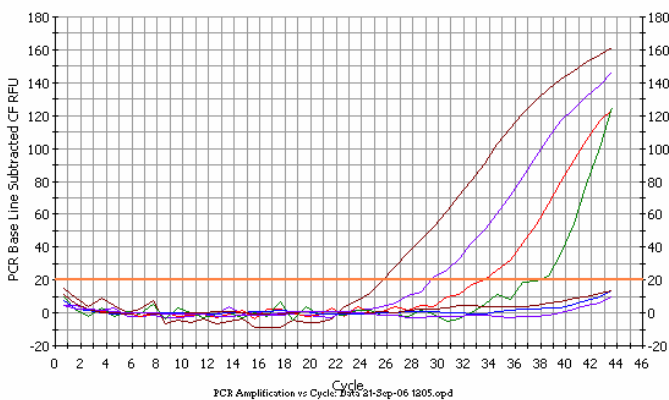
Another attempt was made at the duplex reaction of BRSV/BPI3V with a modification of the concentration of the primers and probe. The concentration of the primers was diluted fourfold and the concentration of the probe was diluted tenfold (this new diluted concentration is the concentration listed in the protocol in the methods section of this paper.) This change seemed to improve the results of the assay as seen below. In addition the annealing temperature was dropped from 60°C to 55°C.

Figure 12.1: **BRSV/BPI3V** 6mM MgSO₄



In the graph above, the least dilute sample is red (lowest c(t) value), the most dilute sample is green, and the negative control is blue.

Figure 12.2: **BRSV/BPI3V** 6mM MgSO₄



In the graph above, the least dilute sample is red (lowest c(t) value), the most dilute sample is also green, and the negative control is blue.

In the next four duplex reactions, the $c(t)$ values do not correlate with the dilution factors of samples making quantification of the viral load difficult.

Figure 12.3: BRSV/BPI3V 5mM $MgSO_4$

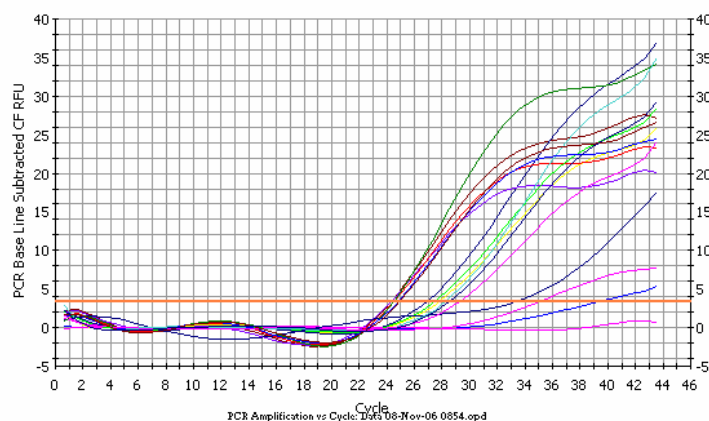


Figure 12.4: BRSV/BPI3V 5mM $MgSO_4$

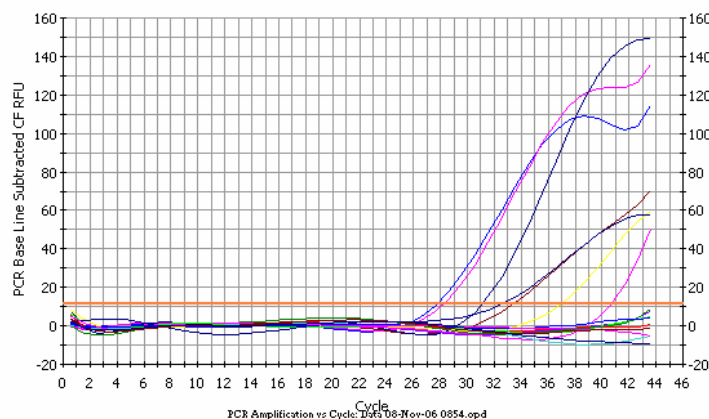


Figure 12.5: BRSV/BPI3V 5mM $MgSO_4$

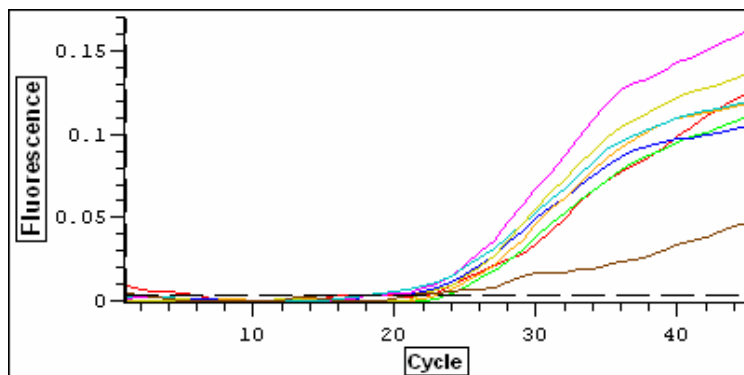


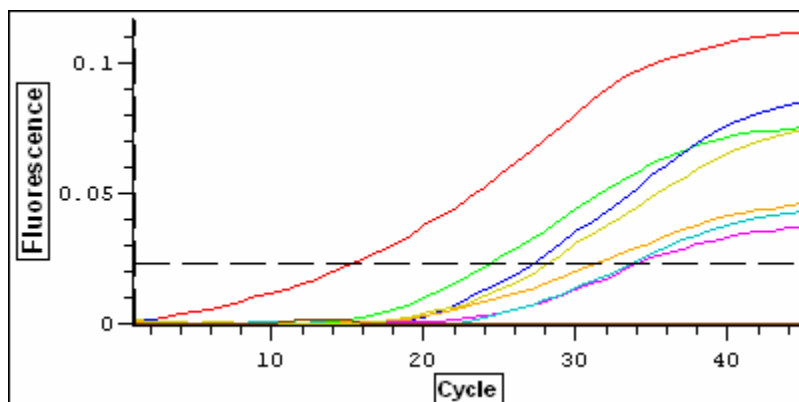
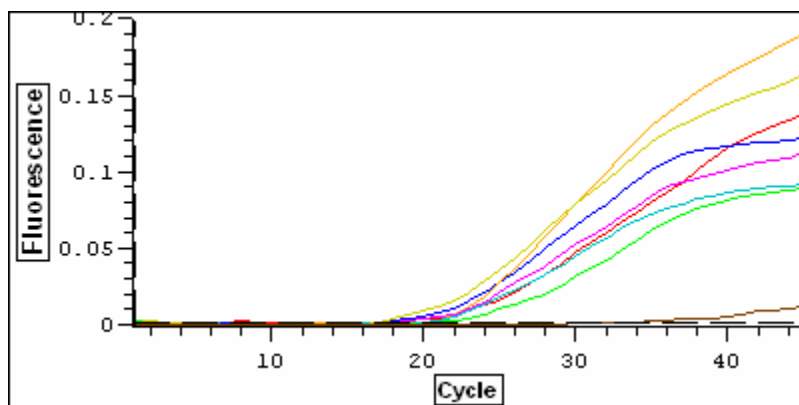
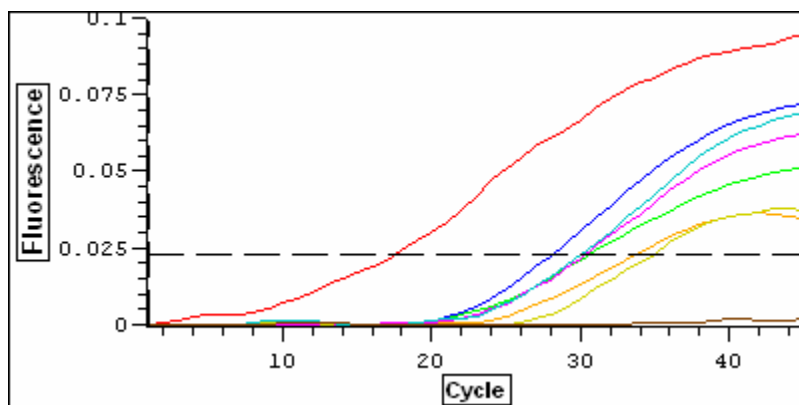
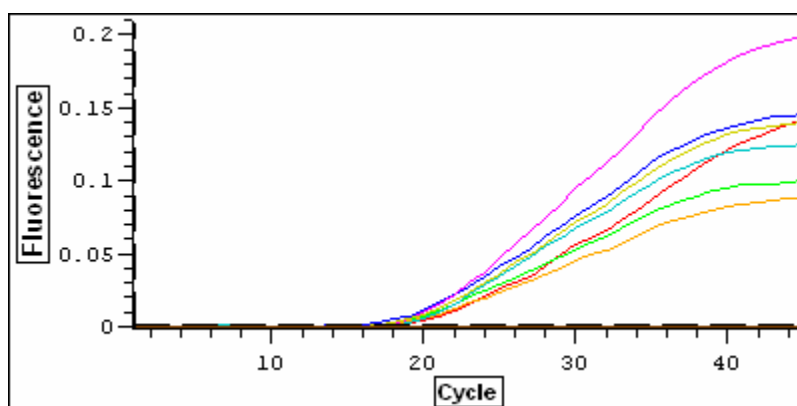
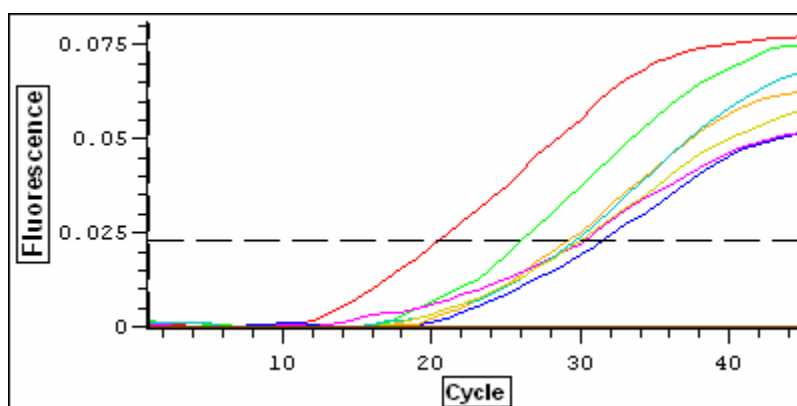
Figure 12.6: BRSV/BPI3V 5mM MgSO₄Figure 12.7: **BRSV/BPI3V** 6mM MgSO₄Figure 12.8: BRSV/**BPI3V** 6mM MgSO₄

Figure 12.9: **BRSV**/BPI3V 7mM MgSO₄Figure 12.10: BRSV/**BPI3V** 7mM MgSO₄

BRSV/BPI3V/BVDV

The following graphs are a multiplex assay screening for all three respiratory viruses simultaneously. Once again, the virus typed in bold face indicates which fluorophore is being read. The protocol of diluted primers and probes, and decreased annealing temperature developed when doing duplex reactions, was continued for this reaction as well. As before, the correlation between the $c(t)$ values and the dilution factor of the samples is not seen as it was with the monoplex reactions. It is therefore, once again, difficult to make any assumptions about concentrations of virus in the samples.

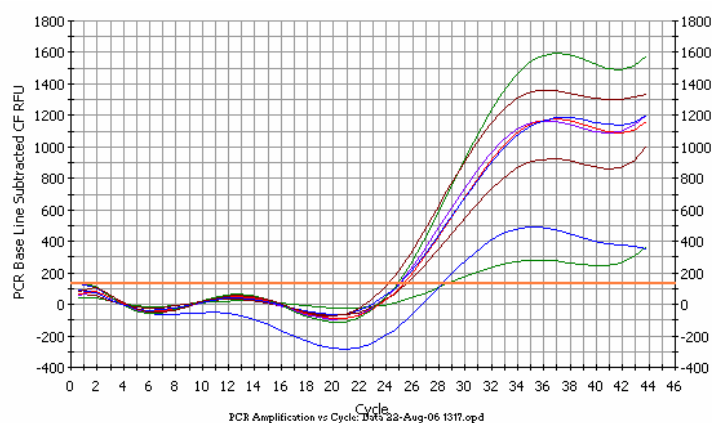
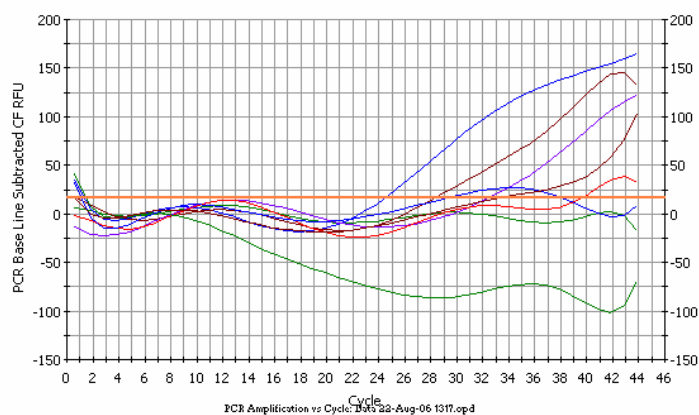
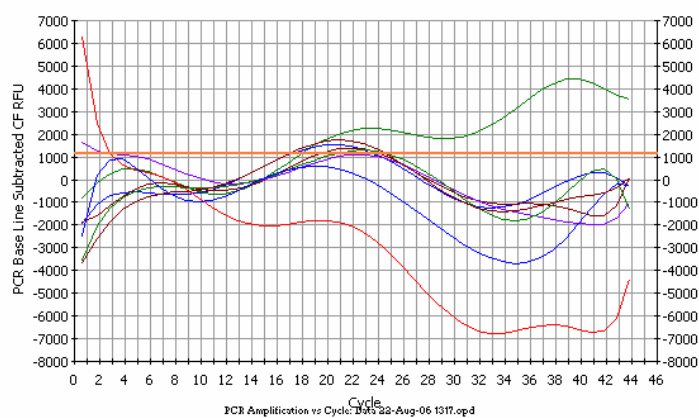
Figure 13.1: **BRSV/BPI3V/BVDV** 6mM MgSO₄Figure 13.2: **BPI3V/BRSV/BVDV** 6mM MgSO₄Figure 13.3: **BVDV/BRSV/BPI3V** 6mM MgSO₄

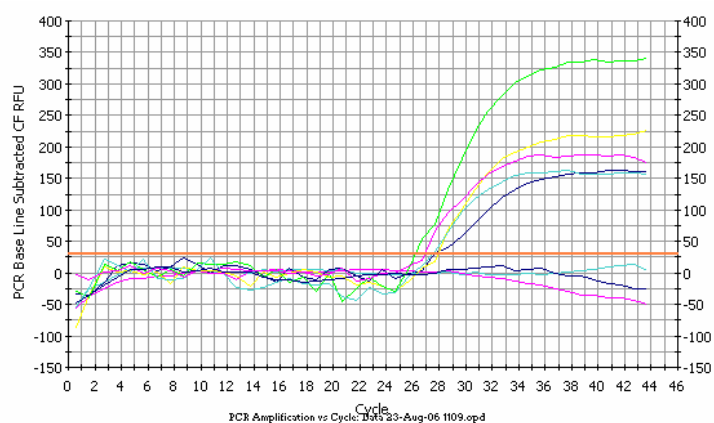
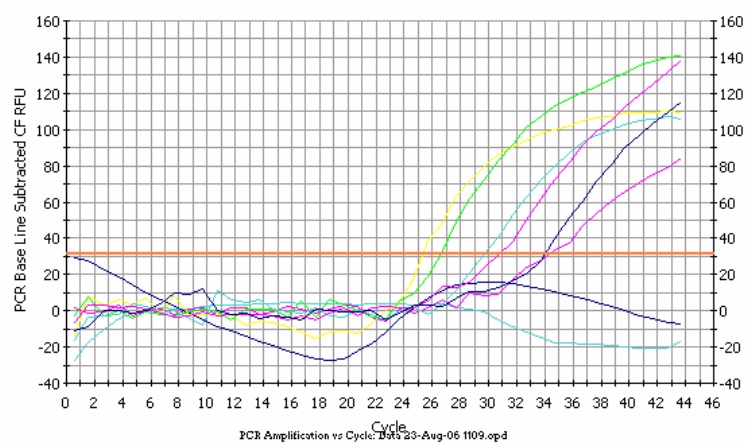
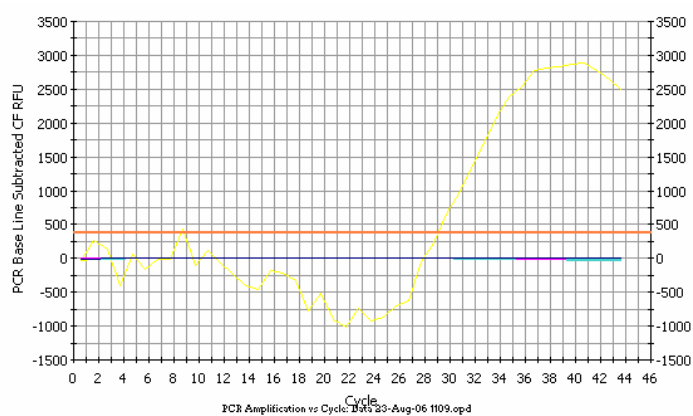
Figure 13.4: **BRSV/BPI3V/BVDV** 5mM MgSO₄Figure 13.5: **BPI3V/BRSV/BVDV** 5mM MgSO₄Figure 13.6: **BVDV/BRSV/BPI3V** 5mM MgSO₄

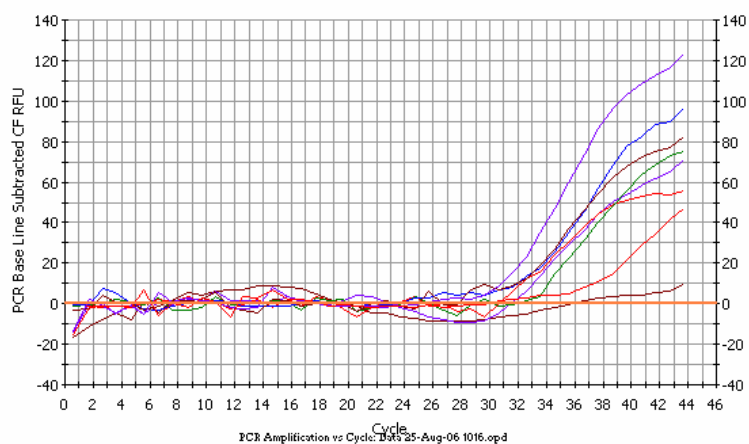
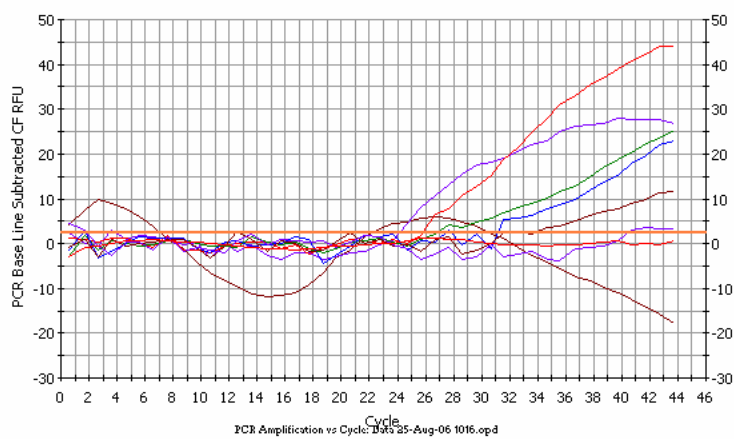
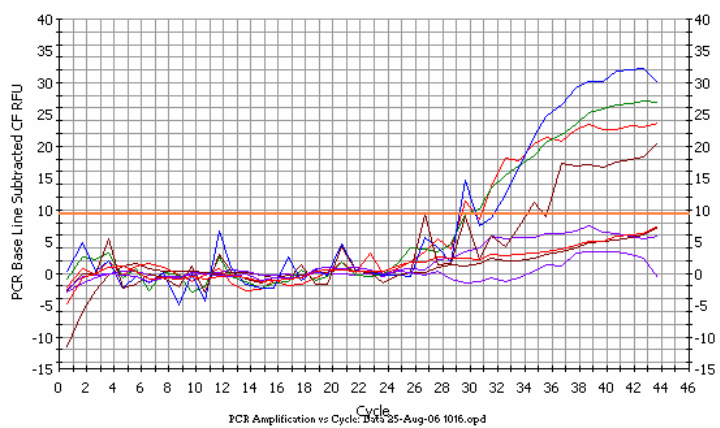
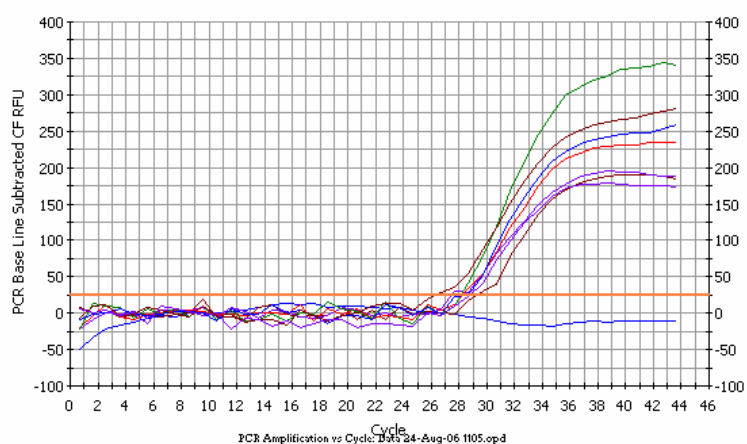
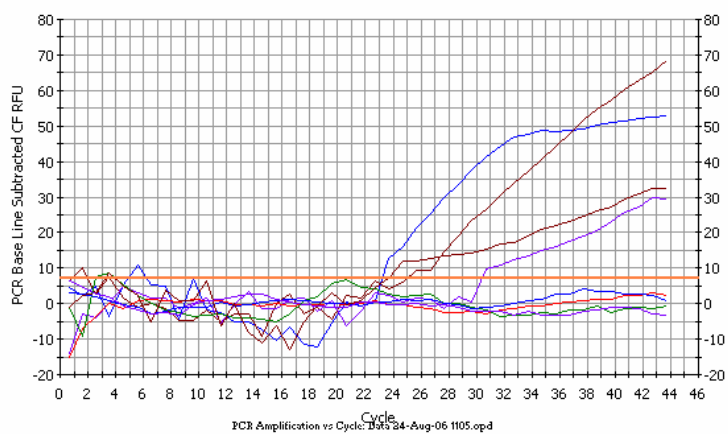
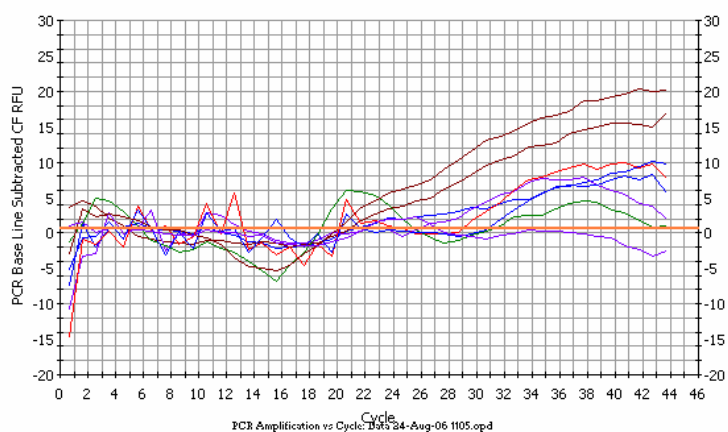
Figure 13.7: **BRSV/BPI3V/BVDV** 4mM MgSO₄Figure 13.8: **BPI3V/BRSV/BVDV** 4mM MgSO₄Figure 13.9: **BVDV/BRSV/BPI3V** 4mM MgSO₄

Figure 13.10: **BRSV/BPI3V/BVDV** 3.5mM MgSO₄Figure 13.11: **BPI3V/BRSV/BVDV** 3.5mM MgSO₄Figure 13.12: **BVDV/BRSV/BPI3V** 3.5mM MgSO₄

Agarose Gel Electrophoresis on real-time multiplex PCR products:

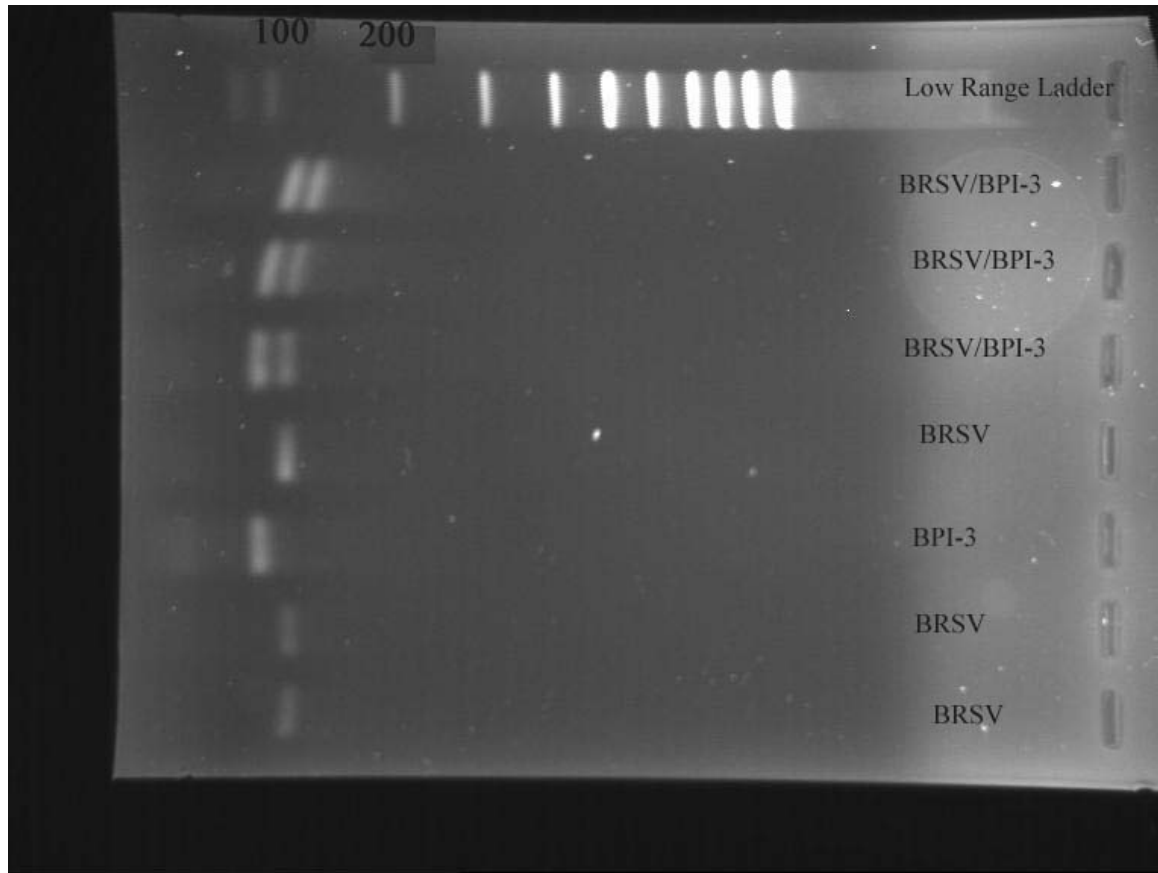


Figure 14: Agarose Gel of monoplex and multiplex assay products. Wells from top: 1) low-range ladder, 2) BPI3V/BRSV duplex, 3) BPI3V/BRSV duplex, 4) BPI3V/BRSV duplex , 5) BRSV 6) BPI3V, 7) BRSV and 8) BRSV.

On the gel there are visibly distinct bands that indicate amplification of the amplicon. The significance of this will be discussed later. This allowed for the next logical step to take place, which was the application of this multiplex assay to multiple respiratory pathogens simultaneously in clinical samples. These results of which will be shown next.

Real-Time Multiplex PCR on clinical samples:

It was determined that not only was the duplex reaction of BRSV/BPI3V the most consistent and reliable of the 4 available multiplexes, but that it was also the assay that would be of most use to the OSU Veterinary Diagnostic Laboratory. Below are the results of the duplex assay, broken into 3 runs for a total of 100 clinical samples. Once again, the clinical samples were run with a MgSO_4 concentration of 5mM.

Figure 15.1: BRSV/**BPI3V** (samples 1-27)

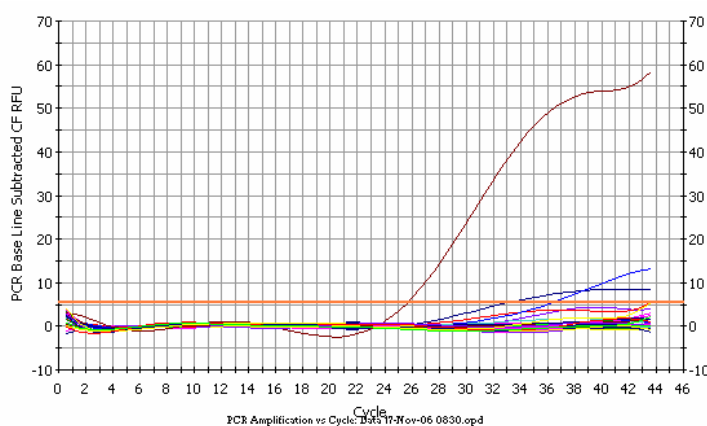


Figure 15.2: **BRSV**/BPI3V (samples 1-27)

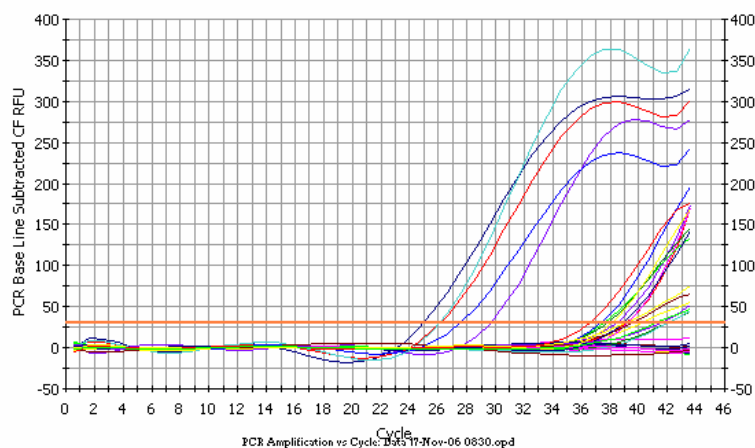


Figure 15.3: BRSV/BPI3V (samples 28-54)

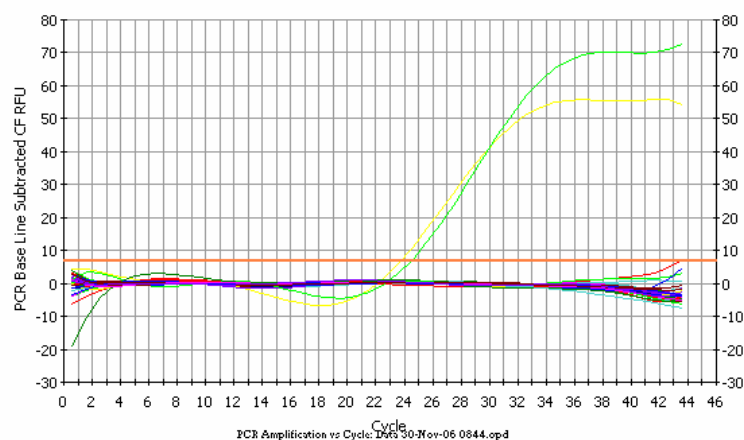


Figure 15.4: BRSV/BPI3V (samples 28-54)

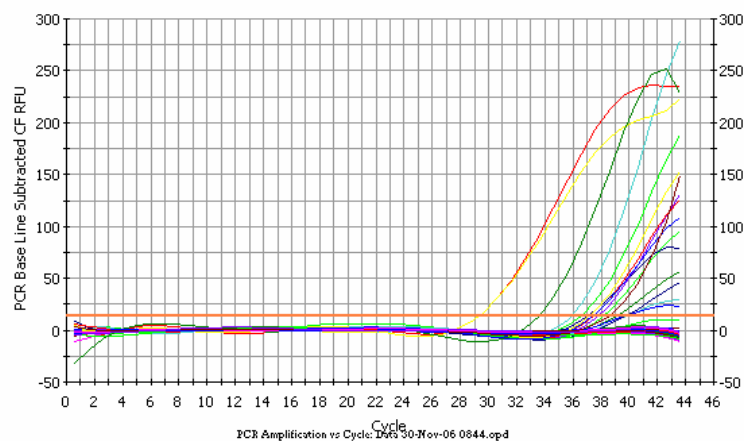


Figure 15.5: BRSV/BPI3V (samples 55-100)

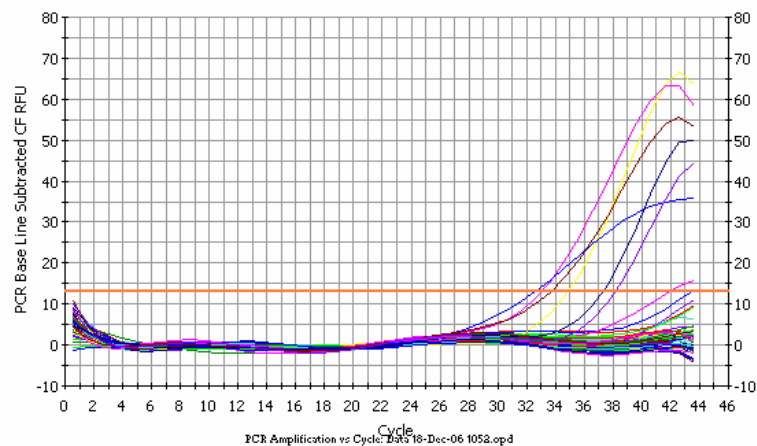
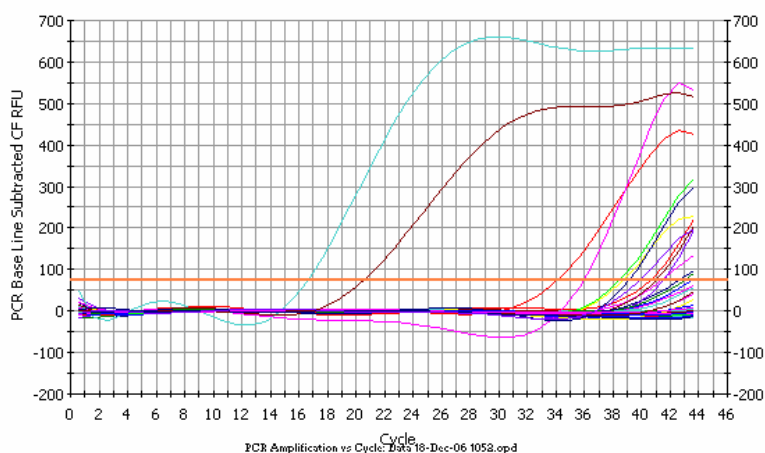


Figure 15.6: **BRSV/BPI3V** (samples 55-100)

Once again, as noted with the results of the monoplex reactions on the clinical samples, there appeared to be a large number of false positives that with ct values greater than 36 and once again, they were counted as negative reactions despite their apparent amplification.

Real-Time Multiplex PCR Clinical Samples Result comparison

Table 4: Clinical sample results.

VDL and PCR results agree
PCR Positive, VDL negative

Sample	BRSV	BPI3V	BVDV	BRSV/BPI3V Duplex	VDL detected
2870	+		+	(+/-)	BVDV
2882		+		(-/-)	
2883				(-/-)	
2884				(-/-)	
2886				(-/-)	BRSV
2928		+		(-/-)	
2962				(-/-)	
2970				(-/-)	
2973				(-/-)	
3029			+	(-/-)	BVDV
3030	+			(-/-)	
3082				(-/-)	
3095	+			(+/-)	

3099				(-/-)	
3100				(-/-)	
3101				(-/-)	
3104				(-/-)	
3105				(-/-)	
3113			+	(-/-)	BVDV
3124				(-/-)	
3125	+			(-/-)	
3126	+		+	(+/-)	BVDV
3127				(-/-)	
3128				(-/-)	
3380				(-/-)	
3383		+		(-/-)	
3395				(-/-)	
3411				(-/-)	
3412				(-/-)	
3413				(-/-)	
3414				(-/-)	
3421				(-/-)	
3430	+		+	(+/-)	BVDV
3431				(-/-)	
3432			+	(-/-)	BVDV
3439				(-/-)	
3440				(-/-)	
3446				(-/-)	
3461				(-/-)	
3472		+		(-/+)	
3488				(-/-)	
3593				(-/-)	
3594				(-/-)	
3595				(-/-)	
3606				(-/-)	
3608				(-/-)	
3610		+		(-/-)	
3611				(-/-)	
3612		+		(-/-)	
3624		+		(-/+)	
3634			+	(-/-)	BVDV
3640				(-/-)	
3653				(-/-)	
3654				(-/-)	
3655	+			(+/-)	
3699	+			(+/-)	
3700	+	+		(+/-)	BRSV
3705				(-/-)	
3707				(-/-)	
3791				(-/-)	
4001				(-/-)	

4002				(-/-)	
4108				(-/-)	
4153			+	(-/-)	BVDV
4156				(-/-)	
4158	+	+		(+/+)	BRSV
4166		+		(-/ +)	
4168				(-/-)	
4179				(-/-)	
4180				(-/-)	
4187				(-/-)	
4188				(-/-)	
6052				(-/-)	
6053				(-/-)	
6055				(-/-)	
6501	+			(+/-)	
6711	+			(+/-)	BRSV
7043				(-/-)	
7770				(-/-)	
7813				(-/-)	
8041				(-/-)	
8045	+		+	(+/-)	BVDV
8048			+	(-/-)	BVD
8049	+			(+/-)	
8102	+			(+/-)	
8103				(-/-)	
8115		+		(-/-)	
8231	+			(+/-)	BRSV
8233	+			(+/-)	BRSV
8304				(-/-)	
8846				(-/-)	
8962			+	(-/-)	BVDV
9481				(-/-)	
9620				(-/-)	
9720			+	(-/-)	BVDV
9952				(-/-)	
9953	+		+	(+/-)	BRSV, BVDV
9954	+		+	(+/-)	BRSV, BVDV
1046				(-/-)	
(+) Control	+	+	+	(+/+)	
(-) Control	-	-	-	(-/-)	

Table 5: Clinical samples results overview.

Run	BRSV	BPI3V	BVDV
Monoplex	19	11	14
Multiplex BPI3V/BRSV	17	4	Not Applicable
VDL	8	Not Tested	14

The 100 samples that were obtained from the OSU VDL were screened for BVDV and BRSV but not for BPI3V. The methods used for screening by the VDL were serum virus neutralization, fluorescent antibody detection and PCR. Of the 100 samples, 8 were determined by the VDL to be positive for BRSV and 14 for BVDV. When running monoplex assays, 19 were determined to be positive for BRSV, 11 for BPI3V and 14 for BVDV. In addition, when running the duplex assay for both RSV and PI3, 17 were determined to be positive for BRSV and 4 for BPI3V. As indicated in Table 4, these results overlapped in that the 8 BRSV positives that the VDL found were also positive in the monoplex and multiplex reactions. Similar correlations were seen with comparisons of the results for BPI3V and BVDV both between the VDL results and the monoplex and multiplex assays. The possible cause for the increased number of positives detected by the monoplex assay will be discussed in the next section.

Discussion

Bovine respiratory disease is a ubiquitous, multifactorial disease that greatly impacts the health of cattle. Many pathogens have been implicated in the causation of BRD complex including both microbial and viral. Because the signs of many of the pathogens mirror each other, it is important to be able to rapidly and accurately differentiate between them so that a proper course of treatment can be determined. The objective of this study was to develop a multiplex real-time PCR treatment for this purpose. While real-time PCR has been proven to be effective for the screening of BVDV (Letellier and Kerkhofs 2003) and BRSV (Achenbach, Topliff et al. 2004), it has yet to be used for BPI3V and multiplex real-time PCR in the veterinary setting is still in its infancy.

Although conventional and real-time PCR has been used previously in the detection of BRSV, the primers and probes for those studies were designed for the F gene, which is not as conserved as the nucleocapsid (N) gene. The primers and probes for this study were designed from a 100% conserved region of the BRSV N gene and from the BIV3V NP gene. The BVDV primers and probes were designed based on a 126-strain nucleotide alignment that amplifies 190 bases. For this reason we expected the results from our assays to be more specific and sensitive than previous methods.

Once the primers and probes were designed, the viruses were cultured as described in the methods section of this paper. These viral cultures were then used to perfect the monoplex and multiplex assays. Over the last year and a half, real-time PCR has been investigated in order to determine its effectiveness in the diagnosis of BRD not only detecting the presence of viral pathogens in the afore mentioned viral cultures but also in clinical samples.

The use of the Ambion MagMAX magnetic bead system for the extraction of the RNA samples proved to be an effective way to isolate the viruses from cell culture. Once the viruses were isolated, real-time monoplex PCR reactions were run for all three viruses. Serial dilutions were made from 10^{-1} to 10^{-7} , and the efficiency of each reaction was determined graphically using Microsoft excel. The impact of the concentration of $MgSO_4$ was noticeable and numerous concentrations were tried ranging from the stock

concentration of 3mM to 6mM. It appeared that the optimal range for this assay for all three viruses was between 4 and 6mM with it peaking at 5mM. The efficiencies of the monoplex reactions for BRSV, BPI3V and BVDV were 96%, 63% and 82%, respectively. While an efficiency of 96% (as seen for the BRSV monoplex assay) was excellent, the efficiencies for the other two reactions were not as high as they should be. Further work will need to be done, especially with BPI3V in order to get more acceptable amplification. The amplification for all three viruses was confirmed, however, using agarose gel electrophoresis, indicating that the assays were indeed successful.

Because of the success of the monoplex assays on the laboratory samples, the next logical step was to perform this assay on clinical samples. The assays for BRSV and BPI3V seem to have a lot of samples turning positive at the end of the run (at cycles greater than 36 in a 45 cycle run) and the cause for this was unknown. This did not seem to be a problem with the BVDV assay. For clarity sake, it was decided that samples with a $c(t)$ value greater than 36 should be considered negative. In addition to the apparent false positives, there were also strong positive reactions (both for clinical samples and positive control samples) and the negative control samples remained negative.

The next logical step was attempting multiplex assays on laboratory samples diluted in the same way as the monoplex assays were. Assays were attempted for each of the following combination of viruses: BRSV/BPI3V, BRSV/BVDV, BPI3V/BVDV and BRSV/BPI3V/BVDV. The duplex reactions were attempted first, to determine which, if any, viruses would cause the most difficulties when combined into a multiplex assay. As with the monoplex reactions, the concentration of the $MgSO_4$ in the master mix was the first part of the protocol that was modified in order to try to improve assay results. After this was tried with little or no improvement, additional changes were made to the protocol, including decreasing annealing temperature, increasing annealing time, decreasing the concentration of primer and probe concentrations, preparation of samples in a sterile environment, and even using different machines and performing repair work on current PCR machines. In general, the results were not as graphically impressive as they were for monoplex reactions. The final protocol that was agreed upon as having the best results is included in the methods section. There appeared to be a lot of interference between primer sequences, which resulted in a lot of noise. Thus, the results that did not

have the logarithmic curve that one would hope to achieve when running this type of assay. Some assays did appear to work better than others. The best assay seemed to be the combination of BRSV/BPI3V. Not only did this assay yield the most consistent results but amplification was verified using agarose gel electrophoresis (page 44). There were distinctive bands for BRSV and BPI3V, not only for the monoplex assays, but also for the duplex assay.

The assay using all three viruses seemed to be plagued with false positives and noise and therefore, was not attempted on clinical samples. The duplex reaction of BRSV and BPI3V, however, was. This was performed with a MgSO_4 concentration of 5mM and had some of the same problems as the monoplex reaction in that there seemed to be a lot of false positives with $c(t)$ values greater than 36. As with the monoplex reactions, these samples were not considered to be positive. However, the negative control samples once again remained negative and the positive control samples showed strong positive results.

As mentioned above, when interpreting the results for the clinical samples, any sample that turned positive after a $c(t)$ of 36 was counted as negative. With this in mind, when looking at the results for the monoplex assays, out of 100 clinical samples tested, 19 were positive for BRSV, 11 for BPI3V and 14 for BVDV. When these samples were collected by the Oregon State University Veterinary Diagnostic Laboratory, they were screened for both BRSV and BVDV. The methods of detection used by the VDL were serum virus neutralization, fluorescent antibody detection and PCR. The VDL found 8 positive for BRSV and 14 positive for BVDV. The samples that tested positive by the VDL also tested positive in the monoplex assays, which indicates an exact 1:1 correlation for the BVDV assay and 11 additional positives for the BRSV assay. Whether this indicates a greater sensitivity for BRSV in this monoplex assay or a greater difficulty with false positives than initially expected is hard to say. Because the samples were not screened for BPI3V by the VDL, no comparisons can be made regarding those results. As for the multiplex reaction, 2 samples that had tested positive for BRSV and 7 samples that had tested positive for BPI3V in the monoplex assays tested negative in the multiplex reaction. This seems to indicate a decreased sensitivity of the multiplex assay compared to the monoplex reaction. These results seem to indicate that while monoplex real-time PCR can be an effective tool in the diagnosis of viral pathogens, multiplex reactions are

still at the beginning stages of development and might not be ready for clinical usage just yet.

Conclusion

Based on the results of this study, it seems that a reliable and effective real-time multiplex PCR assay for the detection of bovine respiratory disease pathogens is still a ways off. It is important to note, however, that the monoplex reactions are effective and did show great advantages over the standard methods of virus detection, including ELISA assays. The multiplex reactions showed evidence of amplification of the viruses as apparent by agarose gel electrophoresis, but the graphical representation of that amplification still leaves the results in question and the interpretation of the graphs is not as clear cut as would be necessary for a definitive diagnosis of BRD viral infection. It is important to also mention that it has now been demonstrated that real-time PCR is an effective method for detecting BPI3V in addition BRSV and BVDV. It will be important in the future to determine how the multiplex reaction can be run more effectively and how to minimize the apparent interference between the various primers so that this assay can get off the ground, because it could prove to be an invaluable assay in veterinary diagnostic medicine. It might be worthwhile to design new primers and probes as an attempt to solve this difficulty. Also, a different kit or machine might show better results than those selected for this study. In addition, it will be important to test this method on more clinical samples in order to confirm its effectiveness in the clinical setting. Once the assay is perfected, it should be a simple matter to add more viruses into the assay, at which point, the only limit would be the number of fluorophores available for testing.

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