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THE EPIDEMIOLOGY OF BOVINE RESPIRATORY CORONAVIRUS INFECTIONS IN FEEDLOT CATTLE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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*****

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ABSTRACT

Bovine coronavirus (BCV) has long been recognized as a cause of neonatal calf diarrhea, and has also been implicated in winter dysentery of adult dairy cattle and outbreaks of calfhood pneumonia. With its tropism for the bovine respiratory tract and the recent isolation of BCV from the upper respiratory tracts of feedlot cattle, BCV has come under suspicion of contributing to the economically significant bovine respiratory disease complex (BRDC) of feedlot cattle. Whether the relationship of BCV with BRDC was causative or merely coincidental was unclear.

In order to define the epidemiology of respiratory BCV (BRCV) infections in feedlot cattle, 1074 calves entering feedlots in Ohio, Texas, and Nebraska over a 3-year period were tested for the presence of BRCV. A BRCV antigen-capture ELISA was adapted to analyze nasal swabs taken throughout the first month on feed, and a BRCV-antibody-detection ELISA was developed to assay acute (arrival) and convalescent (28 days on feed) serum samples. Information was also collected on treatment for respiratory disease, presence of other pathogens, and average daily gain in order to assess the effects of BRCV infection on health and production. BRCV isolates from these feedlot cattle were adapted to cell culture and inoculated into 14 gnotobiotic and 19 colostrum-deprived calves, to determine their pneumoenteric pathogenicity and patterns of viremia.

Surveying these 1074 cattle, entering 7 different feedlots in 12 groups, 7.3% of the cattle were found to be shedding BRCV, with a range of 0 to 35.9% for individual
groups. 94% of the BRCV shedding occurred during the first week on feed. At arrival, 62.4% of the cattle had low (<50) BRCV ELISA antibody titers. Seroconversion to BRCV (defined as a four-fold or greater increase in BRCV antibody titer) was found in 58% of the cattle tested, with ranges for individual groups from 20.3% to 84.1%. Arrival titers to BRCV appeared to be negatively correlated with seroconversion to the virus during the first month on feed.

Health and production data were available on a subset of 838 cattle, data which was used in multivariable logistic regression to assess the effects of BRCV shedding and seroconversion on treatment for respiratory disease, and in multivariable linear regression to determine the effects of BRCV infection on average daily gain. A significant interaction (p=0.06) was found between BRCV shedding and seroconversion, with cattle which were shedding the virus 1.6 times more likely to need treatment for respiratory disease than those animals which neither shed the virus nor developed an immune response to it. Neither BRCV shedding nor seroconversion to the virus exerted any apparent effect on average daily gain (calculated over the length of the feeding period.)

Thirteen BRCV strains isolated from the surveyed feedlot cattle were inoculated into 14 GN and 19 CD calves, which were subsequently monitored for fecal and respiratory shedding of the virus, as well as development of clinical illness. Ten of the 14 GN calves developed diarrhea, 11 of them shed the virus in their feces, and 5 shed the virus in nasal epithelial cells. In the CD calves, 9 developed diarrhea, 6 shed the virus in their feces, and 2 shed the virus from the upper respiratory tract. None of the GN or CD calves developed clinical respiratory disease. Previous inoculation with an enteric (BECV) strain prevented viral shedding and illness following inoculation with a BRCV
strain. Inoculation of BRCV produces diarrhea and viral shedding patterns similar to those seen with BECV inoculation, but does not produce clinical respiratory disease in the absence of other contributory host or environmental factors.

From the available observational and experimental data, it appears that BRCV is capable of contributing to BRDC, but not when acting alone. It most likely acts synergistically with the other infectious agents and environmental stresses feedlot cattle are subjected to, either by suppressing the immune response or potentiating the actions of bacteria such as *Pasteurella hemolytica*. It is possible that preventing BRCV infections via vaccination may help reduce the incidence and severity of respiratory disease in feedlot cattle, but only in conjunction with vaccination for other pathogens and efforts to reduce stress on feedlot calves (i.e., castrating and dehorning calves one month prior to shipment, rather than at arrival.) As with other diseases with multifactorial causes, targeting one agent will not prevent the problem.
Dedicated to my parents, Gael and Kelland
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INTRODUCTION

Changes in disease incidence and prevalence occur due to changes in host, agent, or environmental factors and their interactions with one another. Significant changes over the decades in the methods of producing beef cattle have resulted in increased incidences of diseases that were previously rare or isolated in occurrence. Instead of raising cattle on the same farm or ranch where they were born, cattle are now weaned at an early age and transported long distances, where they are mixed at a centrally-located feedlot with cattle from hundreds of different sources. Although this method of producing high-quality beef is economically preferable to more antiquated means, it does provide a perfect petri dish for the production of potentially fatal pneumonias. Immunologically susceptible calves are exposed to a large variety of infectious agents at precisely the same time they are subjected to a plethora of physical stresses. "Shipping fever," or bovine respiratory disease complex (BRDC) is recognized as one of the most economically significant problems of feedlot managers, and even after years of improved management techniques and therapeutic options, it continues to drain millions of dollars from the beef industry.

A number of viruses, including bovine herpesvirus-1, bovine respiratory syncytial virus, and bovine virus diarrhea virus, are well-recognized contributors to the immunosuppression and pneumopathogenicity associated with BRDC. Incoming calves are now routinely vaccinated against these pathogens, and the search continues for other pieces of the puzzle, additional bacteria or viruses which were previously unrecognized.
as playing a role in BRDC, or which are emerging thanks to immunological selection applied to agents for which vaccines are commonly used. If such agents are recognized as contributing to BRDC, additional prevention and control measures could be established to reduce the incidence of respiratory disease in feedlot cattle.

An example of such an agent of recent interest is bovine coronavirus (BCV). Long recognized as a cause of potentially fatal neonatal calf diarrhea, and associated with outbreaks of pneumonia in young calves (less than 30-days-old), suspicions have arisen that the virus may also contribute to BRDC. A 1996 study confirmed that BCV could indeed be isolated from feedlot cattle suffering from respiratory disease, but as only 100 cattle were sampled, and no healthy controls were included, more questions arose regarding the epidemiology of BCV infections in feedlot cattle. The following study was designed to answer some of these questions.

In order to define the epidemiology of respiratory BCV (BRCV) infections in feedlot cattle in hopes of clarifying its contribution to BRDC, four primary objectives were established for this study:

1.) To develop sensitive, specific diagnostic tests for BRCV antigen and antibodies which would rapidly provide reliable results, allowing the analysis of large numbers of samples.

2.) To survey large numbers of feedlot cattle from a variety of geographic locations for the presence of BRCV, in order to describe the basic patterns of BRCV shedding and seroconversion in populations of feedlot cattle.

3.) To determine the effects of BRCV infection on the incidence of respiratory disease and on production (as measured by average daily gain) in the surveyed cattle.
4.) To determine the pneumoenteric pathogenicity and patterns of viremia of strains of 

BRCV isolated from feedlot cattle, and to compare these characteristics with those of 

previously isolated enteric strains of BCV (BECV).

As with any infectious agent suspected of contributing to a disease complex with 

many component causes, no single study can "prove" that the agent causes the disease in 

question. By describing the incidence and effects of BRCV infection in feedlot cattle, it is 
hoped that either more attention can be focused on preventing and controlling BRCV 

infections, or that the topic can be set aside and resources focused on studying more 

plausible component causes of BRDC.
CHAPTER 1

CORONAVIRUSES AND THEIR ROLE IN BOVINE DISEASE

Coronaviruses are large, single-stranded, positive-sense RNA viruses which can infect a variety of both mammals and birds. With their tropism for epithelial cells of both the enteric and respiratory tracts of many vertebrates, coronaviruses are responsible for a number of economically significant diseases, particularly in newborns. Causing diseases as disparate as transmissible gastroenteritis (TGE) in swine to infectious bronchitis (IBV) in birds (which was the first coronavirus to be isolated and grown in cell culture), to the common cold in humans, coronaviruses were initially grouped into 4 antigenic classifications based on serological evidence. Further analysis, using monoclonal antibodies and nucleotide sequencing, resulted in coronaviruses being divided into 3 antigenic groups. Antigenic group I contains porcine epidemic diarrhea virus, human coronavirus 229E, canine coronavirus, feline coronavirus, and porcine TGE virus. The members of antigenic group II include bovine coronavirus (BCV), murine hepatitis virus (MHV), human coronavirus OC43, porcine hemagglutinating encephalomyelitis virus, and turkey coronavirus. Turkey coronavirus, implicated in the poult enteritis and morbidity syndrome of turkeys, appears to be closely related to BCV, as the majority of monoclonal antibodies produced against either TCV or BCV in one
study could not distinguish between the viruses. The sole member of coronavirus antigenic group III is avian IBV, and isolates of this virus, recovered from the same or different species, can vary widely. In spite of identifiable antigenic differences, coronaviruses share certain morphologic and genomic features regardless of species.

1.1 Coronavirus Structure

Coronaviruses are enveloped, pleomorphic but generally spherical viruses ranging in size from 60 to 220 nm, averaging about 120 nm in diameter. Their name reflects the corona-like fringe of 15-20 nm-long glycoprotein spikes (the S protein) projecting from the spherical surface of the viruses. The S protein ranges in size from 180 to 220 kilodaltons (kDa) and is essential in binding to cellular receptors and inducing membrane fusion. This large protein, being the most accessible portion of the virion, induces the formation of neutralizing antibodies by the host, and often demonstrates more sequence variation within a given coronavirus species than the other 3 structural proteins. In some species of coronavirus, the S protein may be enzymatically cleaved into 2 subunits, the N-terminal S1 and the C-terminal S2, each of which averages 90 kDa. The S2 subunit appears to be moderately conserved between coronavirus species, with up to 30% amino acid homology in certain regions. In contrast, there is almost no conservation of the S1 amino acid sequence between antigenic groups, possessing as it does hypervariable regions of multiple deletions and mutations. Cleavage of the S protein does not occur in all host species, nor is it necessary for cell fusion, and its cleavage depends on the virus strain and the host cells. As the bond between the S1 and S2 subunits is a noncovalent group of basic amino acids, treatment of cells with trypsin
often enhances plaque formation in cell culture when attempting to isolate coronaviruses.

Although most coronaviruses possess only one layer of spikes, bovine coronaviruses (BCV) and other antigenic group II coronaviruses have been shown to have a second, shorter fringe of glycoprotein peplomers, consisting of hemagglutinin-esterase (HE) protein. The HE protein, when present, is 60-65 kDa and, like the S protein, can induce hemagglutination by binding to 9-0-acetylated neuraminic acid residues on the erythrocytes of certain species. The HE protein can also act as a receptor-destroying enzyme, serving as an esterase to reverse hemagglutination by cleaving the acetyl group from 9-0-acetylated neuraminic acid.

The S and HE proteins are embedded in a lipid bilayer envelope derived from the rough endoplasmic reticulum of the host cell. A third structural protein of coronaviruses is the integral membrane (M) protein, which traverses this lipid envelope 3 times and is similar to the matrix protein found in other enveloped viruses. The structural role of the M protein, which ranges from 25 to 35 kDa and is the most highly conserved of the coronavirus proteins, is still unclear, but it is essential for the assembly of virions. The M protein, while not necessary for receptor binding, may be involved in viral pathogenesis, as the M protein of TGEV was found to induce production of alpha-interferon.

Contained within the coronavirus envelope is a ribonucleoprotein (RNP) core, consisting of the RNA genome and a phosphorylated nucleocapsid (N) protein bound to the 5' and 3' ends of the viral RNA. The N protein consists of 3 structural domains and is highly basic. The protein itself is 50- to 60-kDa, and with the RNA forms a helix 14
to 16 nm in length.³ This helix associates with the M protein, forming what was recently revealed, in the case of TGEV and MHV virions, to be an icosahedral core within the virion.¹²

Another recent finding was the existence of a fifth structural coronaviral protein, the small membrane (E) protein, currently identified for IBV, MHV, and TGEV, but not yet identified for BCV.⁷ In these viruses, it is necessary for virus particle assembly, and in addition to the M protein, is the only other viral protein absolutely essential for virion assembly.¹³ The E protein ranges in size from 10 to 12 kDa, and it is postulated that it plays a role in the budding of virions from host cells.¹⁴

In addition to similarities in structural proteins, even antigenically-distinct coronaviruses share some genomic characteristics. The genome of coronaviruses is the largest of all positive-sense RNA viral genomes, consisting of a single-stranded, polyadenylated and capped RNA genome of 27 to 31 kb.³ As the genome is positive sense, the purified RNA of coronaviruses can act as mRNA and is infectious.³ The genome consists of 7 to 10 genes, including the 4 or 5 genes for structural proteins.³ The 5' leader sequence consists of 58 to 90 nucleotides, followed by the gene for the viral RNA-dependent RNA polymerase, needed to produce the complementary negative-strand template.⁷ The general order of the genes, from 5' to 3', following the gene for the polymerase, is: HE, S, M, N, and a 3' untranslated region with a polyadenylated tail.³ A number of nonstructural genes, and open reading frames within genes, varying widely with coronavirus species, are located between the structural protein genes.³
1.2 Coronaviral Replication

Some generalizations can also be made regarding coronavirus replication, which occurs completely in the cellular cytoplasm. Its most striking feature is the formation of a 3'-coterminal nested set of subgenomic mRNAs. The process begins with the binding of the virus to a receptor on the target cell, which is generally an epithelial cell of either the respiratory or enteric tract for most coronaviruses, although some, including porcine hemagglutinating encephalomyelitis virus and certain MHV strains, can also cause neurologic disease. With a few exceptions, coronaviruses usually infect only their natural host species. In most cases it is the S protein that binds to 9-O-acetylated neuraminic acid residues on glycoproteins or glycolipids on the cell surface, although in the case of BCV the HE protein may also bind to these moieties. The exact mechanism of virus entry into the host cell after receptor binding, whether by endocytosis or by acidic pH-nondependent pathways, is unclear, and appears to depend on the specific virus and host cell strains. Following virion entry into a host cell and subsequent uncoating of the genome, the RNA coding for the RNA-dependent polymerase is translated, which then transcribes a full-length negative strand template. As the coronavirus RNA-dependent RNA polymerase, unlike its viral DNA-dependent counterpart, has no proof-reading capability, error rates in transcription can be as high as 1 in $10^4$. Recombination may be one way to balance the errors caused, resulting in constant change and the production of antigenic diversity. Another property contributing to the high recombination rate is the ability of coronavirus RNA to crossover more than once within a genome.
Once the negative-sense template is available, the 5' leader RNA, as well as genomic and subgenomic mRNAs, are transcribed from this negative-sense template. The leader RNA serves as a primer for the polymerase further down the template, reassociating with intergenic regions of the RNA to produce the "nested" set of one genomic and six subgenomic mRNAs, all of which are polyadenylated, contain the 5' leader sequence, and share 3' sequences, differing in the sequence near the 5' end. It is this unique 5' sequence which is translated, producing the structural and nonstructural proteins. The mRNAs for the structural proteins are mostly monocistronic, whereas the mRNAs for nonstructural proteins are polycistronic, producing up to 3 proteins from the overlapping open reading frames of each mRNA.

Following post-translational processing of the proteins in the Golgi complex, including glycosylation and cleavage of the S protein in some host species, the N protein binds to the RNA genome, forming the RNP core. This core associates with the other proteins and the components of the viral envelope, with virion assembly beginning in the perinuclear region but moving eventually to the rough endoplasmic reticulum. The assembled virions travel through the Golgi complex from cis to trans, where they are released from the cell by fusion of virus-containing vacuoles with the apical plasma membrane of the infected cells. It appears that the small E protein plays a crucial role in determining the site of viral budding from the host cell.

The effects of viral replication on the host cell vary according to strain, and will be discussed in particular for strains of BCV. It is possible that coronaviruses cause cell death via apoptosis, as they can cause lytic and persistent infections. In 1993 Levine et al. demonstrated that the sindbis virus establishes lytic and persistent infections by
triggering apoptosis of host cells. Other viruses, including adenoviruses and bovine herpes virus type 1, have also been shown to induce apoptosis during the infection of host cells. Coronaviruses may cause cell death in a similar manner, but to date, no studies have confirmed or denied this.

1.3 Bovine Coronavirus

BCV, a member of antigenic group II of the coronaviruses, was first recognized as a potentially fatal cause of neonatal calf diarrhea in 1972. Extensive investigations since that time have also implicated BCV in winter dysentery in adult dairy cattle, as well as respiratory infections of cattle of varying ages. The virus appears to be ubiquitous in cattle populations, as serum antibodies to BCV can be found in most adult cattle throughout the world. Coronaviruses antigenically identical to BCV have also been isolated from wild ruminants, including white-tailed deer and mule deer. Previous investigations have expanded the body of knowledge concerning BCV, and raised additional questions regarding its role in other disease processes, particularly the respiratory disease complex of feedlot cattle.

Using negative-stain electron microscopy, a coronavirus-like agent was first identified in calf feces in 1972. The virions were 65 to 210 nm in size, possessing a fringe of 25-nm-long peplomers. As the major properties of this agent were like those of members of the Coronaviridae family, it was classified as bovine coronavirus. Like the other members of this family, BCV is an enveloped, positive-sense, single-stranded RNA virus. It is composed of 4 major structural proteins, the 190 kDa S protein, which is cleaved into 90 and 110 kDa products, a disulfide-linked HE dimer of 120-140 kDa, an
M protein of 25 kDa, and an N protein of 50 kDa, which can form disulfide-linked trimers.\textsuperscript{10}

Replication of BCV follows the pattern previously described for coronaviruses, wherein the virus attaches to the host cell using both the S and HE proteins\textsuperscript{29}. The virus enters the cell by direct fusion with the host cell plasmalemma,\textsuperscript{30} not requiring an acidic intracellular compartment for entry.\textsuperscript{31} All replication takes place in the cytoplasm, beginning with the transcription of an RNA-dependent RNA polymerase, which then directs the transcription of a negative-sense template.\textsuperscript{10} From this template, one full-length genomic RNA and 7 subgenomic mRNAs are produced, all of which share 3′ ends.\textsuperscript{10} The unique regions of these mRNAs are translated, the products modified, then the virions assembled by budding within both the rough endoplasmic reticulum and the Golgi apparatus.\textsuperscript{10} Generally the virions are released by fusion of virus-filled vacuoles with the apical plasma membrane, although some may also be released by the lysis of host cells.\textsuperscript{10} The potential role of apoptosis in BCV pathogenesis has not yet been delineated.

1.4 Neonatal Calf Diarrhea

BCV’s tropism for absorptive epithelial cells leads to severe, potentially fatal diarrhea in very young calves, usually those from 3- to 30-days old,\textsuperscript{20,28} although cases in calves as old as 3 months have been reported.\textsuperscript{10,20,32} The virus attacks cells throughout the length of the small and large intestines, starting with the proximal small intestine, but histopathological lesions are most often found in the lower small intestine, the large intestine and cecum.\textsuperscript{33} Affected calves become depressed and develop a profuse, watery diarrhea. As the infected tall columnar epithelial cells on the tips of the
intestinal villi undergo pyknosis, karyorrhexis, and lysis, they are replaced by immature cuboidal and squamous epithelial cells from the crypts. BCV may also induce apoptosis in host cells, as is the case with viruses such as bovine herpes virus-1 and Sindbis virus, but no in vivo studies have yet confirmed this. The crypt epithelium becomes hyperplastic, and the lamina propria is infiltrated by plasma cells and lymphocytes. The villi become shortened and club-shaped, with a villous:crypt ratio of 1:1 to 2:1, and adjacent villi may fuse. This villous atrophy and fusion greatly reduces the surface area and absorptive capacity of the gut, a problem which is compounded by the presence of immature crypt cells, with their increased secretory activity and diminished ability to produce needed digestive enzymes. The volume of fluid in the gut increases, and the presence of undigested lactose increases adverse microbial activity, creating an osmotic imbalance and drawing still more fluid into the gut. Water and electrolytes are lost, leading to progressive dehydration, acidosis, hyperkalemia, hypovolemia, and hypoglycemia, which can be fatal, usually within 24-48 hours of the onset of diarrhea.

On necropsy, undigested milk is found in the abomasum, and the intestinal tract is thin, transparent, and distended with a clear fluid. Petechiation may be present on the intestinal mucosa. The treatment of BCV enteritis is supportive and non-specific, consisting of fluid replacement therapy and possibly kaolin to reduce the severity of the diarrhea. If infected calves survive, the diarrhea will resolve in 5 to 6 days.

The diarrhea caused by BCV in calves is often indistinguishable from that caused by other pathogens, including enterotoxigenic E. coli, rotavirus, Cryptosporidium, Salmonella, bovine viral diarrhea virus, and combined infections. Enteric BCV
infections can be diagnosed using electron microscopy (EM) or immuno-electron microscopy (IEM) to identify virus particles in intestinal contents or in frozen sections of intestine. However, correct identification of coronaviral particles can be difficult, as cellular debris and fragmented bacteria and mycoplasma can also present as fringed spheres that closely resemble BCV. The specific identification of BCV in feces and intestinal contents was improved using protein A-colloidal gold immunoelectron microscopy (PAG-IEM), which accurately identified BCV but did not label morphologically similar fecal debris. Other methods of BCV diagnosis include fluorescent-antibody (FA) examination, passive hemagglutination, and enzyme-linked immunosorbent assays (ELISAs) utilizing monoclonal antibodies. Virus isolation can also be used as a diagnostic tool, but as BCV can be difficult to grow in cell culture, this method is usually not a practical option for most diagnostic laboratories.

Utilizing these diagnostic methods, BCV has been commonly found in both healthy and diarrheic calves, with incidence rates of 0 to 24% for healthy calves, and 8 to 69% in diarrheic calves. Although more commonly identified in dairy calves, BCV enteritis also occurs in beef herds. Due to the heat-labile nature of BCV, enteritis outbreaks are more common in winter months, and can often occur year after year on the same farm. The virus may be transmitted through either the fecal-oral route or via respiratory transmission, with calves being exposed to the virus from other infected calves, the environment or subclinically infected adult cattle within the herd. Studies have found BCV antigen in the feces of 70% of clinically normal adult dairy cattle, in spite of the presence of BCV antibodies, indicating these adults could provide an excellent reservoir for the persistence of BCV in a herd. In light of the persistence of
BCV in carrier animals, and the economically significant morbidity and mortality caused by infection of neonates, investigations were designed to understand the immune response to BCV infection and the potential for protective immunization.

1.5 Immune Response

Experimental oral or intranasal inoculation of 4 gnotobiotic calves with BCV resulted in IgM antibody peaks in both the feces and nasal secretions at day 7, after which these antibodies declined to relatively low levels. Nasal and fecal IgA antibody levels peaked after IgM levels did, by post-inoculation day (PID) 10 to 14, and were undetectable by PID 21 to 35. The same pattern of antibody response was seen in the serum, but the IgA antibodies were not present for as long a duration as observed in the feces and nasal secretions. In most of these calves, fecal and nasal viral shedding, as assayed by immunofluorescence (IF) and IEM, diminished with the rise in antibody titers. IgG1 antibodies were not detected in the serum until PID 14, and IgG2 antibody responses in most calves were not detected until PID 21.

These patterns of antibody response were confirmed and expanded by continued investigations. Five colostrum-deprived calves were inoculated orally and intranasally with BCV during the first 30 hours of life, then challenge exposed with BCV 21 days later. Blood, nasal swabs, feces, saliva, tears, and bronchoalveolar lavage (BAL) fluids were collected from each calf and analyzed for the presence and type of BCV antibodies using an indirect double-antibody sandwich ELISA and an immunoblot assay. It was found that IgM antibody, directed primarily at the N protein of BCV, was the first isotype to appear in the feces and mucosal secretions, seen first during post-infection week (PIW)
1. This response was followed by the appearance of IgA antibodies, directed against all 4 major BCV proteins, during PIW 2 and 3.

Following the challenge dose of BCV, IgA antibody titers increased, or failed to decrease, in all mucosal secretions, except for IgA antibodies directed against the N and M proteins in the feces. The IgG antibody titers to the S protein, in tears and BAL fluid, and to the HE protein in BAL fluid, also increased following the challenge exposure. The serum antibody responses to BCV were similar to those seen in other studies, beginning with a significant IgM antibody increase, directed against the N and HE proteins, followed by the production of IgG1 antibodies against all viral proteins. The IgG2 and IgA antibody responses were slower and of smaller magnitude. Following administration of the challenge dose of BCV, serum levels of IgG1 antibodies to all proteins either increased or failed to decrease. Additionally, serum IgA antibody titers against M, S, and HE proteins, IgG2 responses to the S and HE proteins, and IgM antibody titers to the N protein also increased or failed to decrease following the PIW 3 challenge exposure.

A similar investigation was undertaken to determine immune responses to BCV in calves in field situations, and to evaluate the correlation of these responses with protection against BCV infections. Blood, feces, tears, and nasal swabs were collected 3 times per week from 2 groups of 10 calves each in a large closed dairy herd in Ohio, from birth up to 9 to 20 weeks of age. Incidences of enteric or respiratory illness were reported. The samples were analyzed using ELISAs to quantitate the antibody titers and to detect BCV antigen. All of the calves in the first group suffered at least one occurrence of diarrhea or fever, and 8 out of the 10 were shedding BCV, according to
IEM analysis of fecal samples. 45 Seven out of 10 calves in the second group developed diarrhea at least once, and 9 out of the 10 experienced fever at least once. 45 Four of these calves were shedding BCV from either the respiratory or intestinal tract. 45 As in the experimental BCV challenges, these calves demonstrated strong IgM antibody responses prior to 2 weeks of age, followed by IgA, IgG1, and IgG2 antibody responses in the first 4 weeks of life. 45 BCV antibodies, primarily IgM, IgA, and IgG1, were found in the feces of all calves at one week of age, most likely due to the continued feeding of colostrum on this farm. 45 Another study revealed that the feeding of colostrum with high titers of BCV antibodies increased the levels of passive IgG1 and IgA antibodies in both the serum and mucosal secretions of calves, but delayed or decreased the active IgG1, IgA, and IgM antibody responses to BCV in serum and secretions, as compared to calves fed low-titer colostrum. 46

The protection of neonatal calves against BCV infection can be enhanced by either improving lactogenic immunity or by vaccinating them with an attenuated live virus, to stimulate an active immune response. 2 Intramuscular vaccination of dams with a live, attenuated BCV did not increase antibody levels in either milk or colostrum, whereas use of an inactivated virus, or intramammary administration of the virus, did increase the protective titers of both milk and colostrum. 2 Although it is apparent that lactogenic immunity, in the form of serum-derived IgG1 antibody in cattle, 47 is crucial for the protection of neonates against infection with BCV, complete protection can interfere with the development of active immunity, following either natural exposure to the virus or administration of a vaccine. 48 An alternative to enhanced lactogenic immunity is the use of oral vaccines in calves. In both field and experimental conditions,
however, oral vaccines were shown not to be effective in protecting against BCV infections. Investigators were successful in stimulating BCV immunity in newborn calves using *in utero* vaccination with an attenuated virus. Unfortunately, this method of vaccination resulted in unacceptably high rates of abortions and premature births.

Although vaccination of calves and/or dams may not be highly effective in preventing BCV enteritis, other control measures can be instituted on farms suffering from recurrent outbreaks. As with other calfhood diseases, incidence can be reduced through good management procedures, including: regularly cleaning maternity pens, removing calves from their dams shortly after parturition, administration of sufficient amounts of colostrum to all calves, provision of clean, dry calf hutches which prevent nose-to-nose contact between calves, thorough disinfection of shared equipment, and training of personnel in biosafety procedures.

One of the most significant challenges in reducing the incidence of BCV infections in calves is the ubiquitous presence of BCV in adult cattle. Although many healthy cattle may shed the virus, creating a risk for neonates and susceptible herdmates, the virus is not always a benign presence in its adult host, as seen in the case of winter dysentery (WD).

### 1.6 Winter Dysentery

For over 60 years an acute diarrheal disease was recognized in both beef and dairy cattle. Affected cattle suffered from semi-liquid, bloody diarrhea and a dry cough, with a marked reduction in milk production. Although the disease could be severe in susceptible individuals, and result in an attack rate of 100%, it was rarely fatal, and was seen most commonly from November to March in the northern United States. Similar
outbreaks of adult cow diarrhea during the winter months were reported by Rollinson in England in 1948. Over the next 20 years, reports of a winter diarrheal syndrome continued to accumulate, from Sweden, Canada, Australia, and the United States. These reports were all similar in their descriptions of the pathology and epidemiology of WD, but varied in the hypothesized cause of the disease.

Necropsies of both naturally and experimentally infected (using feces from an affected individual) cattle provided needed information on the pathology of the disease, but did little to shed light on its cause. Common findings among these necropsies included: inflamed abomasal mucosa, segmental catarrhal inflammation of the jejunum and ileum, injected serosal vessels, and petechial hemorrhage of the spiral and distal colons. Typical histopathology of affected animals included pyknosis, granular degeneration, hydropic and hyaline degeneration, and karyorrhexis of mucosal epithelial cells on both the villi and in crypts.

It was apparent that this disease was infectious and contagious, and could be produced by inoculation of susceptible animals with feces from infected animals. The specific causative agent or agents proved difficult to identify. For many years *Campylobacter fetus* subspecies *jejuni* (formerly *Vibrio jejuni*) was considered the cause of WD, due to isolation by Jones of vibrio organisms from the diarrheic feces of calves infected with feces from affected cows. Other investigators were unable to isolate either vibrio organisms or *Salmonella* species from affected dairy cattle, nor could they readily identify any bacteria, mycoplasma, or protozoa which could be causative.

Shortly after this, evidence began to accumulate implicating BCV in the cause of WD. Horner and coworkers, using EM, found coronavirus-like particles in feces from
dairy cows affected with WD in New Zealand. Coronavirus-like agents were also found in diarrheic feces from adult dairy cattle in Japan, and in samples from 6 beef and dairy herds in France, in the absence of other viruses, spirochetes, or *Salmonella* species. Using immunohistochemistry, Van Kruiningen found coronavirus-like agents within the crypt epithelial cells of spiral colons of affected cattle from 2 Connecticut dairies, and lesions similar to those of BCV-associated neonatal calf enteritis.

A 1987 outbreak of WD in a closed Ohio dairy herd added to the evidence pointing to BCV as the WD causative agent. Twenty out of 136 lactating animals were affected, and no evidence of enterotoxigenic *Eschericia coli*, *Salmonella* species, coccidia, *Cryptosporidia*, or BVDV was found. Using IEM and PAG-IEM, coronavirus particles were found in the feces of 8 of 9 samples from sick cows, and in none of the samples from clinically normal herdmates. Inoculating gnotobiotic calves with fecal filtrates from cows with WD, a coronavirus was isolated from the calves' feces, which was then successfully propagated in cell culture and found to be antigenically and morphologically similar to the Mebus strain of calf diarrhea BCV. An additional study by Saif et al. found coronavirus in feces from cattle in 6 herds affected by WD, but did not detect coronaviral particles in any samples from 3 herds suffering from non-hemorrhagic diarrhea. Antibody seroresponses, as defined by a fourfold or greater rise in BCV neutralizing antibody titer, were detected in all of the WD affected herds, but were not found in any members of the non-WD herds.

Improvements in diagnostic technology, including IEM and reverse transcriptase polymerase chain reaction (RT-PCR), and continued interest in WD provided more and more evidence implicating BCV in WD. However, BCV infection in WD herds has
not been proven consistently, as BCV has not been found in all WD herds tested, and other viruses, including rotavirus, have been found in WD herds. As shedding of BCV most likely occurs early in the course of WD and is transient, it may be present and not detected. It is also possible that WD is caused by multiple agents, or is not correctly diagnosed due to the lack of a clearly specified case definition for WD. WD can resemble many other cattle diseases, including acute BVDV infection, salmonellosis, and parasitism, emphasizing the need for prompt and accurate diagnostic testing in suspected cases.

Continued studies of WD revealed the contributions of certain host and environmental factors to disease development. Older cattle appear to have acquired immunity to the disease, as the most susceptible cattle have been reported to be recently fresh 2- to 6-year-old cows. Recent parturition or poor nutrition may also increase the severity of the disease. Previous infection may enhance immunity to WD, as the disease could not be produced in steers that had been infected with filtered virus 3 months prior to the challenge dose. Cross-protective immunity to BCV was demonstrated by El-Kanawati by inoculating gnotobiotic calves with WD strains of BCV, then cross-challenging them with heterologous CD and WD isolates. A recent study by Fukutomi et al. revealed a high degree of sequence conservation between CD and WD strains isolated in Japan, but some antigenic diversity due to amino acid substitutions in the polymorphic region of S1 subunits. Herd-level immunity may also be a factor in WD outbreaks, as investigators have noted that whole regions experience the disease simultaneously, but that some herds in these regions escape infection. However, the role of herd immunity is debatable, as one study found that herds with low immunity (no
WD in the past 4 years) had the highest attack rates, yet another study found that previous WD incidence increased the risk for recurrent infections. A recent Ohio study found that recent herd exposure to BCV appeared to increase that herd’s risk of WD.

Additional factors are suspected of playing a role in the causation of WD. Using manure-handling equipment to handle feed, increased prevalence of seroconversion to BVDV, and tie-stall housing rather than freestalls are herd factors found to contribute to an increased incidence of WD. Within herds affected by WD, open cows are more likely to be ill than pregnant ones, as are animals with high acute BCV antibody titers. Environmental factors also contribute to the incidence of the disease, with numerous investigators linking close confinement and poor ventilation of barns with occurrence of WD. A recent study by Tsunemitsu et al. found that both CD and WD strains of BCV are capable of causing diarrhea in adult dairy cattle, in association with environmental and/or host factors. RT-PCR revealed BCV in the diarrheal feces from these experimentally-inoculated cattle. Ongoing studies seek to further define and understand the interactions between host and environment in the cause of WD, and the role of BCV in its causation.

1.7 Calfhood Pneumonia

During investigations of enteric BCV, both in calves and adult cattle, it was noted that BCV could also cause respiratory infections in cattle. The first report of BCV involvement in a respiratory disease outbreak came in 1982, when Thomas and co-workers conducted a microscopic and pathologic survey of 8 outbreaks of naturally occurring pneumonia in calves in Great Britain. In order to identify any previously unrecognized causes of respiratory disease (defined as coughing, fever, nasal discharge,
and inappetance) in calves under 6 months of age, serum samples and nasopharyngeal swabs were taken from 8 calves in each of the 8 outbreaks. Virus isolation, immunofluorescence, serology, and EM were used to analyze the samples for the presence of microorganisms. Lung washes and nasopharyngeal swabs were then used to inoculate 18 gnotobiotic calves. Most of the sampled calves yielded organisms previously associated with calfhood pneumonia, including adenovirus, parainfluenza-3 (PI-3), and BVDV. However, in calves from 2 of the outbreaks, coronavirus particles were found in lung wash and nasopharyngeal samples, using EM and subsequent isolation in bovine tracheal cultures. The isolated virus reacted with antiserum to an enteric strain of BCV.

Other investigators confirmed the tropism of BCV for the respiratory tract, growing the virus in fetal bovine tracheal organ culture, nasal mucosa epithelial cell culture, and bovine embryonic lung cultures. In 1984 McNulty et al. isolated 2 coronaviruses, one from the lungs of a 12-day old Holstein calf with gross and histologic evidence of pneumonia, and the other from a 3-to-4-week old Holstein calf with lung consolidation and mild tracheitis. After propagation of these isolates, for 3 to 6 passages, in bovine tracheal organ culture and confirmation as BCV using immunofluorescence with an enteric anti-BCV conjugate, the isolates were inoculated intratracheally and intranasally into 7 calves. All calves were less than a week old, and 5 of the 7 were colostrum-deprived.

Nasal mucus and nasopharyngeal swabs were taken every 2 days throughout the course of infection, from 5 to 10 days. These samples, as well as the respiratory tissues taken at necropsy, were tested for the presence of BCV using IF. Four of the 7
calves developed either coughs or nasal discharge, but labored breathing or elevated rectal temperatures were not observed. Four of the 5 colostrum-deprived calves developed diarrhea within 1 to 3 post-inoculation days, and were found to be shedding large numbers of BCV in the feces. In all 6 of the calves where nasal mucus was IF tested for BCV antigen, viral shedding was found throughout the course of infection. Coronavirus antigen was also found in the lungs of 2 of the calves, as well as several areas of superficial lung collapse in the cranial lobes. No other common respiratory pathogens, including PI3, BVDV, and bovine respiratory syncytial virus (BRSV) were found in the lungs from these calves, suggesting the lesions were due to BCV.

Evidence suggesting a possible role of BCV in calfhood respiratory disease continued to accumulate. In a 1985 study, Reynolds et al. obtained feces and nasopharyngeal swabs from 69 dairy calves with diarrhea and 46 clinically normal calves, and tested these samples for the presence of BCV using IF and ELISAs. BCV was found in 23% of the feces from diarrheic calves, and in 20% of the nasal swabs from the same animals. No BCV was found in the feces from normal calves, and only 4% of the normal calves yielded BCV-positive nasal swabs. A significant (p<.001) association was found between the simultaneous fecal and nasal shedding of BCV in the diarrheic calves. Twelve of the calves sampled, known to be suffering from severe diarrhea, were necropsied. BCV antigen was detected via IF in both intestinal and respiratory tissues in 8 of the calves, in the colon only in one of the calves, and in respiratory tissues alone in 3 of the calves.

Thirteen gnotobiotic calves were inoculated with BCV isolated from either the respiratory or intestinal tracts of calves involved in these outbreaks. All 13 calves
suffered infection of both the enteric and respiratory tracts, and although all calves developed some degree of diarrhea, no clinical signs of respiratory disease were observed. Nasal swabs taken from these calves were positive for BCV from the first day after inoculation for a mean period of 5.3 days. Four of these calves were challenged a second time, 12 to 21 days after the initial inoculation, and their patterns of viral shedding compared to those of 3 age-matched, previously uninfected control calves. The previously-infected calves were immune to challenge with the homologous virus, whereas the control calves did show evidence of viral replication. Although BCV antigen was not found in the lower respiratory tracts of any of the experimentally-challenged calves studied, and they did not develop respiratory disease, this investigation did support the possibility of BCV aerosol transmission and replication in the respiratory tracts of calves.

A 1986 study by Saif et al. used gnotobiotic and colostrum-deprived calves to evaluate the effect of route of inoculation of an enteric BCV isolate on viral replication, distribution, and time course of infection in both the enteric and respiratory tracts, and clinical course of disease. Eighteen calves, eleven 3- to 50-day old gnotobiotic calves and seven 25-to 63-day-old colostrum-deprived conventional calves, were divided into 3 groups and inoculated with a fecal BCV isolate. The 8 calves in group 1 were inoculated orally/intranasally, the 5 calves of group 2 were inoculated orally, and the 5 members of group 3 were administered the virus only intranasally. Fecal specimens and nasal swabs were collected each day, and tested for the presence of BCV using IEM or IF staining (for the nasal epithelial cells). Fourteen of the calves were euthanized on
postinoculation days (PID) 3 to 7, and evaluated for the distribution of BCV antigen in the respiratory and intestinal tracts using direct IF staining.

The calves did not develop signs of respiratory disease, but did develop profuse diarrhea between PID 2 and 4. All but 2 of the calves (which had been inoculated orally only) shed BCV in the nasal epithelial cells, and all calves shed BCV in their feces. The shedding patterns were affected by the route of inoculation, with all but 1 of the orally infected calves shedding virus first in their feces, followed by nasal shedding. Virus shedding was detected first in the nasal epithelial cells in most of the intranasally inoculated calves, followed by fecal shedding, whereas calves inoculated both orally and intranasally were found to be shedding virus in the feces and nasal cells concurrently.

Upon necropsy, all of the 14 calves examined had BCV antigen in the intestinal tract, particularly concentrated in the ileum and colon. Three of the orally inoculated calves yielded BCV antigen in cells in the upper respiratory tract, and 4 of the 18 calves (one orally inoculated, one intranasally challenged, and 2 challenged via both routes) had BCV antigen in their lungs. Two of these calves had had elevated rectal temperatures during the course of infection, and had focal interstitial emphysema in their lungs at necropsy. From these findings, it appears possible that a mild upper respiratory tract infection with BCV, in a calf immunologically compromised by concurrent BCV diarrhea, could permit the colonization of the lower respiratory tract by other respiratory pathogens, leading to pneumonia.

Although these studies delineated the course of experimentally-induced BCV respiratory infections, more data was needed to understand the nature of the virus' ability to infect the respiratory tracts in a field setting. In 1990 Heckert et al. performed a
prospective longitudinal study of 8 calves, from birth to 120 days of age, in 2 dairy herds in Ohio. Rectal swabs and feces, as well as nasal swabs, were collected periodically from each of the calves, and rectal temperatures and clinical signs were recorded three times per week throughout the study period. Nasal epithelial cells and supernatant fluid were tested for the presence of BCV using IF staining and ELISAs, and viral shedding in the feces was analyzed using IEM.

During the test period, 6 of the 8 calves (75%) shed BCV, either via the intestinal or respiratory tract, at some point during the 4 month survey. Six of the calves developed diarrhea, 3 of 8 had signs of upper respiratory disease, and all had elevated rectal temperatures at least once. As measured by ELISA or IEM, the occurrence of diarrhea could be associated with BCV shedding in 3 of the calves, and respiratory disease as associated with BCV shedding (as measured by IF or ELISA) in 2 of the calves. The respiratory shedding of BCV occurred beginning at 6 and 9 weeks of age, and fecal BCV antigen shedding began at 5, 9, and 16 weeks of age. Serum antibody responses to BCV also occurred in both young and older calves, with 3 calves under the age of 30 days and 4 calves over 30 days in age demonstrating increases in serum BCV antibody titers. No significant correlations were found between total serum antibody levels and clinical disease, nor between clinical disease and increases in specific BCV antibody isotypes. The results of this study indicated that both enteric and respiratory BCV infections could occur in calves over a month old, and that they may be common, although often subclinical, in a closed herd.

Another study conducted by the same investigators found similar results in another large (>800 head) dairy herd, where 2 groups of 10 calves each were sampled
from birth through 10 to 20 weeks of age, as described previously. In the first group, 7 of the 10 calves had rhinitis at least once, and all calves in this group had BCV respiratory tract infections, according to IF results on nasal epithelial cells. The average age of the calves when rhinitis was first noted, in association with respiratory BCV shedding, was 14 days, with a range of 5 to 19 days. In the second group, 2 calves were shedding BCV via the respiratory tract, but the incidence of rhinitis in this group was unassociated with the shedding of BCV. From this field study, it was found that BCV respiratory tract infections were common on this farm, and that most of the initial infections occurred between the ages of 1 and 3 weeks. A strong correlation was found between isotype antibody titers to BCV and the number of respiratory sick days, further strengthening the hypothesis that BCV replication in the upper respiratory tract may predispose calves to infections with other respiratory pathogens.

Although these studies established that BCV can infect the epithelial cells of the upper respiratory tract of cattle, its potential to infect the lower respiratory tract is still under investigation. There is evidence to suggest that BCV can infect the lungs, as in the previously-mentioned 1986 study which found focal emphysema in calves experimentally infected with BCV, as well as BCV antigen. In 1991 Kapil et al. orally infected 3 calves with a virulent pneumoenteric BCV isolate, finding that the number of nasal cells positive for BCV on direct immunofluorescent microscopy increased from 0.1% on PID 2 to 20% by PID 7. Two of these calves showed gross lung pathology at the time of death, including cranioventral interstitial pneumonia, congestion, and hemorrhage. Samples of lung tissue from both calves were IF-positive for BCV. In a 1995 study, Stine et al. oronasally inoculated 4 gnotobiotic calves with a respiratory
BCV isolate from adult cattle. Three of the 4 calves developed bronchiolitis, bronchiolar necrosis, and mild interstitial pneumonia, as seen at necropsy at PID 4-10. Whether or not diarrhea was induced was not reported. Immunohistochemical staining detected BCV in the lungs and nasal turbinates of one of these calves, and in the colon and ileum samples of all 4 calves. 82

1.8 Comparisons of Enteric and Respiratory BCV Strains

Evidence indicates that BCV, like its porcine counterpart, infects both the intestinal and respiratory tracts. There is also evidence that some strains are quite similar in their antigenic and biologic properties. Gnotobiotic calves infected with enteric calf BCV (BECV) strains were resistant to challenge with certain calf respiratory BCV (BRCV) strains. 79 Antisera to 9 different BECV and BRCV strains effectively neutralized the virus in homologous sera, and cross-reacted with the 8 other isolates. 79 In another investigation, a cross-neutralization test was used to compare 14 isolates from calf feces and nasal swabs with the Mebus Nebraska calf diarrhea coronavirus as well as 2 of the 14 isolates. 23 Each antiserum neutralized the Mebus strain and the isolates equally, lending support to the hypothesis that the strains are antigenically similar. 23

However, there is also evidence indicating that some antigenic and biologic diversity exists even between BECV strains. In virus neutralization tests, antisera to 1 neonatal calf diarrhea (NCD) and 2 winter dysentery (WD) strains of BECV had 16-fold or lower antibody titers against 3 WD and 1 NCD strains than against the homologous strains. 83 As this variation reflected low antigenic relatedness (R) values, it suggests the presence of different subtypes among BCV. 83 In hemagglutination inhibition (HI) tests, some one-way antigenic variation among strains was also observed, suggesting that
antigenic and biologic diversity exists among BECV strains, but is unrelated to the source
calf or adult cow diarrhea of the strains.

A recent study by Hasoksuz et al. compared 10 recently isolated respiratory
strains of BCV with previously isolated WD and CD BECV strains, which had previously
been shown to belong to at least 2 distinct antigenic subtypes. Based on HA and RDE
activity, 6 of the BRCV strains were similar to 2 CD and 2 WD strains of BECV. The
other 4 BRCV isolates differed from the other BRCV strains and the BECV strains and
were classified into a distinct antigenic subtype. Nine of the 10 BRCV isolates were
similar to the 2 BECV subtypes based on VN and HI results, but one strain was not
similar to either BECV or BRCV isolates.

Additional differences between the BECV and BRCV strains may also exist at the
molecular level, as they do between transmissible gastroenteritis virus (TGEV) and
porcine respiratory coronavirus (PRCV) strains. The molecular basis for tissue
tropism differences between these strains may be due to a deletion in the S glycoprotein
gene, which is present in PRCV strains but not TGEV strains. An initial comparison
of the nucleotide sequences of the S gene of the BRCV strain G95, isolated from nasal
swabs of a calf with clinical respiratory disease, with an enteropathogenic strain, BECV
LY138, detected 98.7% nucleotide and 98.3% amino acid sequence homology. No
deletions or insertions were found, and nucleotide substitutions appeared to be randomly
distributed. However, S protein-specific monoclonal antibodies differentiated between
BRCV G95 and the BECV strain L9, but did not differentiate between G95 and BECV
LY138, suggesting that genomic comparisons between more divergent strains may be
needed to reveal molecular differences. Alternatively, molecular differences, like
antigenic differences, may vary among BCV strains, and may be unrelated to the clinical source of the isolate.

1.9 Bovine Respiratory Disease Complex

It is clear from both experimental and field studies that BCV can infect and replicate in the upper respiratory tracts of calves, and may also infect the lower respiratory tract. This natural tropism leads one to consider the possibility of BCV infecting the respiratory tracts of older cattle, and possibly contributing to respiratory disease in older cattle, not just calves. Respiratory disease is recognized as one of the most financially significant causes of morbidity and mortality in the cattle industry. It is estimated that the cattle industry loses between $168 to $624 million a year to respiratory disease. In a 1991 National Agricultural Statistics/APHIS survey of cattle and calf deaths, respiratory disease accounted for 31% of the total deaths. The feedlot industry in particular continues to search for means to prevent and control bovine respiratory disease, due to the heavy financial burden it inflicts on their feedlot industry annually. In a 1996 review of feedlot records from the central United States, Edwards found that 67 to 82% of the total morbidity in feedlots was due to respiratory disease. A 1994 study by Vogel and Parrott revealed respiratory disease as the cause of 44.1% of the deaths in Great Plains feedlots. Not only does respiratory disease result in readily identifiable costs, such as the cost of antibiotic treatment and death loss, but more subtle expenditures can also be attributed to respiratory disease, such as reduced average daily gain, prolonged days on feed, and the cost of personnel needed to monitor pens for sick individuals and treat sick animals.
Known as bovine respiratory disease complex, (BRDC) or shipping fever, pneumonia in feedlot cattle has plagued feedlot managers for as long as cattle have been kept in closely confined feed yards. Numerous investigators, over several decades, have studied the problem of BRDC, what causes it, and how best to prevent it. It is a difficult disease to prevent, due to its multiple causes and their complex interactions with one another. Generally, it is accepted that the innate immunologic defense mechanisms of the respiratory tract are overwhelmed by a combination of viral infections, including bovine respiratory syncytial virus (BRSV), bovine herpes virus-1 (BHV-1), parainfluenza-3 (PI-3) and bovine viral diarrhea virus (BVDV), and multiple stressors, including transportation, mixing with cattle from different sources, inclement weather, and rapid changes in ration. With the normal immune response suppressed, normal bacterial inhabitants of the upper respiratory tract, including Pasteurella hemolytica and multocida, can now infect the lower respiratory tract and cause fibrinous bronchopneumonia, leading to illness, production losses, and death.

A number of studies have been performed on substantial numbers of feedlot cattle in order to determine the significance of various infectious agents and environmental factors in contributing to BRDC. A landmark study of BRDC was the Bruce County Beef Project, conducted by Martin et al. in 1978, 1979, and 1980, on 52,889 calves entering feedlots in Bruce County, Ontario. Not only was this study notable in its scope, but it was also one of the first veterinary investigations to look beyond infectious agents to possible multicausal theories of disease causation. Data were collected on every aspect of each operation, including management variables (will cattle be finished to market weight?), demographic variables (will cattle be mixed with others from different
lots?), ration variables (corn silage vs. hay silage, use of starter rations), and processing variables, such as treatment with prophylactic antibiotics, vaccinations, and dehorning. Using percent mortality and treatment costs as measures of illness, stepwise multiple regression analysis was used to determine the effect of the selected variables on the occurrence of illness in cattle within the first 6 to 8 weeks after arrival at the feedlots. 

Mortality rates each year ranged from 1% to 1.3%, with pneumonia being the most common cause of death, causing 49% to 63% of the deaths in cattle examined. Fibrinous pneumonia, caused by *Pasteurella hemolytica*, was the most frequent postmortem diagnosis. Approximately 29% of the cattle were treated at least once. Feedlot owners spent $1 per head on preventive measures, and $3 per head on treatment costs. Several factors were found to contribute to increased mortality rates among groups of cattle. Cattle being fed to fatten during the first winter had higher mortality rates than those being fed only for growth, and groups of cattle that had been extensively mixed had higher mortality rates and higher treatment costs than cattle that had been kept together as lots. Other variables contributing to higher mortality rates and increased treatment rates included feeding cattle corn silage as the major part of the ration within a week of arrival, the use of respiratory vaccines, dehorning within 2 weeks of arrival at the feedlot, feeding hay silage, and the use of prophylactic antimicrobials in the water supply. Although these findings did offer insight into how management could be altered to reduce mortality rates and treatment costs, they did not solve the puzzle of BRDC, as it continued to plague even producers who made the recommended changes.

More information regarding the infectious organisms responsible for BRDC was still needed. A serologic survey was conducted from 1983 to 1985 to determine the
presence of 7 suspected respiratory pathogens in Ontario feedlot calves and the effect of seroconversion on the incidence of respiratory disease. 

Blood was drawn at arrival, and again at 28 days after arrival, from 279 calves identified as suffering from respiratory disease (as determined by the feedlot manager) and another 290 “healthy” control animals. None of the calves was vaccinated for any of the organisms until after the study had been completed, nor had they received vaccines prior to arrival at the feedlot. 

The samples were tested for the presence of IBR, BVDV, BRSV, and PI3 using either serum neutralization (SN) or hemagglutination inhibition (HI) tests. Antibodies to Mycoplasma bovis, M. dispar, and P. hemolytica were detected using an indirect hemagglutination test, and the response to the P. hemolytica cytotoxin was measured via toxin neutralizing activity.

The investigators found that over half the arriving cattle had detectable titers to P. hemolytica, its cytotoxin, and both Mycoplasma species, 40% had titers to BVDV, and fewer than 25% of the cattle had titers to IBR, BRSV, and PI3. During the first 5 weeks following arrival, respiratory disease accounted for 90% of all cattle requiring treatment. After the first month on feed, more than half the calves seroconverted (defined as a four-fold or greater increase in titer) to PI3, BRSV, and the Ph cytotoxin, and about 40% of the calves seroconverted to P. hemolytica, Mycoplasma, and BVDV. Very few animals (<5%) seroconverted to IBR. Initial titers were moderately to strongly negatively correlated to subsequent titer changes for each organism, but titer changes between organisms were independent. Several factors were found to increase the risk of treatment for respiratory disease, including a less-than-average weight, and seroconversion to Ph cytotoxin, BRSV, and BVDV. Seroconversion to these organisms
was predictive of respiratory disease cases, accounting for almost 69% of all respiratory
disease cases in the feedlots. Titors to *P. hemolytica* and IBR at arrival were predictive
of respiratory disease, while higher PI3 and BVDV titers at arrival appeared to be
protective. However, when analyzed on an individual animal basis, weight gains and
relapses of BRDC were not strongly related to initial antibody titer, or to seroconversion
to any of the tested organisms.

As cattle in feedlots are housed and managed as groups, it is only logical to
analyze feedlot serology data at the group level. Using the same groups of calves from
which the cases and controls were drawn in the previous study, Martin et al. tested 879
calves in 14 groups. The data was tested for associations, at the group level, between
serological titers to *P. hemolytica* surface antigens, Ph cytotoxin, IBR, BVD, PI3, BRSV,
*M. dispar*, *M. bovis*, and respiratory disease treatment rates, relapse of disease, and 28-
day weight gains. Using least squares regressions, it was found that seroconversion to
BVDV and *M. bovis* was associated with increased respiratory disease rates, while
seroconversion to *P. hemolytica*, Ph-cytotoxin, *M. dispar*, and PI3 were predictive of
decreased weight gains. As in the individual-level analysis, titers were not predictive of
relapses of clinical respiratory disease. The only organism to which seroconversion
was predictive of respiratory disease at both the individual and group levels was BVDV.
This study further supported the hypothesis that infection with multiple agents is
common in BRDC, and that analysis should be conducted at the group level when
studying feedlot data.

In order to better define the roles of the suspected respiratory pathogens tested for
using serological methods, another study was undertaken by Allen et al. in 1992 to
determine the association between antibody titers to these organisms and the bacteriological and cytological evidence of their presence in feedlot calves. A group of 136 calves, similar to those used in the previous studies, was observed for their first month on feed at an Ontario feedlot. Calves showing signs of respiratory disease were assigned to a group of cases, and were matched with an apparently healthy control. Before receiving treatment, nasopharyngeal swabs, bronchoalveolar lavage (BAL) fluid, and blood samples were taken from the cases and the controls. Calves were bled again 12 days later to provide a convalescent serum sample. The swabs and BAL were cultured for bacteria and mycoplasma, and the BAL samples were examined cytologically. Sera were analyzed for the presence of antibodies to BHV-1, BVDV, PI3, BRSV, *P. hemolytica*, and Ph cytotoxin.

Serological evidence of exposure to all the organisms was found to be common, except for BHV-1, to which titers were found in only 2 cases and 2 controls. The prevalence of acute titers to all other organisms, and their mean values, did not differ significantly between cases and controls. Similarly, convalescent titers to all organisms except PI3 and *P. hemolytica* were not significantly different between case and control groups. For these 2 organisms, convalescent titers were significantly higher in controls than cases. Rates of seroconversion, as defined by a four-fold or greater increase in titer, were similar in cases and controls, except for seroconversion to PI3 and *P. hemolytica*, which were found to have a protective effect in regard to respiratory disease. Nor were any significant differences found in the number of agents to which each calf seroconverted.
Studying the BAL fluid samples, investigators found cytological evidence of pulmonary inflammation in both cases and controls, and found cytologically-normal samples in calves from both groups. A positive association was found between seroconversion to *P. hemolytica* and its cytotoxin, and the isolation of *P. hemolytica* from BAL. Cases were found to be positively associated with the presence of neutrophils and *P. multocida* in the BAL. Multiple linear regression analysis was used to determine if any associations existed between seroconversion, and the presence of macrophages or neutrophils in the BAL samples. No significant associations were found. Evaluating the serological, cytological, and bacteriological evidence from calves in both groups, it was found that it was difficult to distinguish cases from controls based on this data, as almost 60% of the respiratory disease which occurred was not explained by results from the analysis of the available data. It was likely that other factors, such as management or environmental factors, or agents not tested for, played a significant role in determining susceptibility to respiratory disease. It was also possible that a large number of subclinical infections occurred in the supposedly healthy control animals, further clouding the difference between healthy and sick animals. As the convalescent samples were taken only 12 days after the acute samples, a large number of primary antibody responses may have been missed, biasing the results toward those animals which were demonstrating a secondary antibody response to BCV.

In addition to studying the effects of infectious agents on the incidence of BRDC, it was clear that a better understanding of potentially contributory environmental factors was also needed. In a study conducted on 58,885 spring-born calves entering a large feedlot in southwestern Alberta from September 1 to December 31 from 1985 through
1988, Ribble et al. examined the effects of time of year, weather, and patterns of auction market sales on the incidence of BRDC. Cases were defined as animals with clinical depression, a temperature over 40.5°C, and the absence of clinical signs referable to a system other than respiratory. Necropsies were performed on all mortalities in order to remove ambiguity concerning cause of death. Mortality due to fibrinous pneumonia was the outcome variable for the models used in this study.

Ribble found that the number of feeder cattle marketed on a weekly basis was very consistent from year to year, starting in the first week in August and running through December, with the greatest proportion of calves being sold during the last week in October and the first week in November. The number of calves purchased by the feedlot followed the supply of calves available at the 42 auction markets in the 4 western Canadian provinces used by the feedlot’s order buyers. Each year, the maximum risk for developing respiratory disease occurred 2 to 4 weeks after the peak in calf sales at the auction markets. It was found that calves entering the feedlot in November were 2 to 8 times more likely to develop fatal fibrinous pneumonia than calves entering the feedlot in September or December. This pattern was consistent from year to year. The authors hypothesized that several factors contributed to this pattern, including changes in calf density at the auction markets, feedlot population dynamics that altered feedlot crew efficiency (with more animals entering the feedlot, there would be less time to observe individuals for signs of illness), and changes in transport truck availability.

Weather data, including mean monthly temperature, total monthly precipitation, and number of days per month with measurable precipitation, were collected from the Calgary Weather Office and a local weather station. It was found there were no annual
differences in weather patterns that coincided with changes in risk for pneumonia, although the records did indicate November was the month with the greatest drop in daily temperature, and there were more days with measurable precipitation in November than December. The authors felt that data from many more years, and from more than 1 feedlot, would be needed to more accurately assess the effect of weather patterns on the risk for BRDC.

It was clear from this study that the risk of BRDC increases significantly as the fall progresses and feedlots fill with calves, but it was still unclear what other factors may contribute to this increased risk. Ribble, et al. used data from the same group of calves, entering the same Alberta feedlot from 1985 to 1988, to examine the effects of transportation and pretransit mixing on the risk for BRDC. As BRDC was traditionally known as shipping fever, it has long been recognized that transporting cattle over long distances to feedlots may contribute significantly to the development of BRDC. However, with the change from rail transport to trucks, the length of time cattle were confined, and restricted from eating and drinking, decreased dramatically, as the trucks allowed calves to be moved hundreds of miles from farm to feedlot in hours, rather than days. Ribble and his coworkers found no correlation between the distance a group of calves was transported and their subsequent risk for fatal pneumonia, or shrinkage at arrival. The risk of fatal fibrinous pneumonia was the same in calves from nearby markets as it was in calves transported over 1000 kilometers.

Using this same data set, Ribble also evaluated the effect of extensive mixing, prior to transportation to the feedlot, on BRDC risk. Due to the large size of the feedlot, order buyers would purchase numerous small groups of calves at 42 different auction
markets, then assemble these calves into larger groups to fill a truckload. Most farms contributed only 1 or 2 calves to each truckload, with a median of 2 calves/farm in a truckload, a median which was consistent over all 4 years. As the degree of mixing of pens of cattle at the feedlot varied little from year to year, it could not completely explain the wide variations in risk for BRDC. However, when truckloads were grouped according to order buyer, the risk for BRDC and the mean number of calves per source were negatively correlated. It is thus likely that feedlot managers should more aggressively observe and treat groups of calves that were highly mixed prior to transport, but, as in the other studies, this variable was found to be one of many contributing to BRDC, none of which can be singled out as the “cause” of BRDC.

The search continues for ways to predict and/or prevent BRDC. Wittum et al., in a 1996 study, analyzed serum haptoglobin concentrations in feedlot cattle, in an effort to predict clinical respiratory disease. High levels of haptoglobin, an acute-phase protein produced by the liver following infection or inflammation, have been associated with numerous infectious conditions in cattle, including metritis, mastitis, pericarditis, and traumatic reticulitis. Determining the levels of haptoglobin in feedlot cattle may therefore indicate animals suffering from early or subclinical pneumonia, allowing treatment prior to significant production losses. Serum samples were obtained from 366 Nebraska beef calves at arrival, and again at 40 and 65 days on feed. Records were kept on signs of clinical respiratory disease, and 144 of the calves were examined for signs of BRDC at necropsy.

Similar proportions of calves had detectable levels of haptoglobin at each of the 3 sampling times, and 58% of the calves had detectable levels of haptoglobin in at least 1
Following the day 40 sample, the serum haptoglobin concentration increased as the proportion of clinically ill calves increased. After 65 days on feed, it was found that calves with a high level of serum haptoglobin (>10 mg/dl) had a higher rate of clinical respiratory disease than those calves with lower concentrations. At necropsy, only 39% of calves without detectable serum haptoglobin were found to have gross pulmonary lesions, whereas 63% of those calves which had had measurable amounts of serum haptoglobin, in at least 1 of the 3 samples, were found to have gross pulmonary lesions. Unfortunately, even though associations were found between serum haptoglobin responses and respiratory disease, the magnitude of the associations limited the usefulness of haptoglobin response as a predictor of respiratory disease.

Similar results were obtained in a study designed by Wittum et al. to quantify the haptoglobin response to respiratory tract disease, using 60 affected feedlot calves. Following diagnosis with respiratory disease, calves were randomly assigned to either a control group, or a group receiving antibiotic treatment. Although calves in both groups had similar serum haptoglobin concentrations initially, calves that received antibiotic treatment had lower haptoglobin levels after recovery. Haptoglobin concentrations in control calves decreased only slightly from initial diagnosis to recovery. No statistically significant differences in haptoglobin concentrations at initial diagnosis were found between calves with gross pulmonary lesions at slaughter and those without. It appeared that haptoglobin response was quite variable, and while it may serve as a measure of response to antibiotic treatment, it was unrelated to the severity of the case and the need for treatment.
Although the causes of BRDC remain difficult to define and untangle, its effect on production is clear. In trials on calves in their first 28 days, differences of .14 kg to .23 kg in ADG were found between calves that suffered from BRDC and those that did not. In another trial, encompassing 90 days on feed, those calves which developed BRDC gained an average of .18 kg less per day than healthy calves, and those which relapsed had an ADG of .33 kg less than their healthy cohorts. Traditionally, it was thought that calves which were ill initially would undergo a “compensatory gain,” in which they would gain even more weight than healthy calves, following recovery. The results of this study demonstrated the absence of a compensatory gain in the case of BRDC, as groups of calves started the feeding period at similar weights.

The adverse effects of BRDC are not limited to the first weeks on feed. In a study of cattle fed from 138 to 235 days, Bateman et al. found that those cattle treated for respiratory disease had a 0.06 kg lower ADG at the end of the feeding period than cattle which had never received treatment for BRDC. Similar results were found in the 1992-1993 and 1993-1994 Texas A&M Ranch-to-Rail studies, wherein calves treated for respiratory disease had 0.09 kg lower ADG than untreated calves. Treatment costs for respiratory disease in these studies ranged from $20.76 to 37.90 per head treated, and the calves which were treated were then worth $0.19 to $0.35 less per kg than healthy calves.

The true costs of BRDC are difficult to completely quantify, especially in light of the prevalence of subclinical infections. In a study published in 1996, Wittum et al. followed 469 steers from birth to slaughter, monitoring them for clinical respiratory disease and average daily gain. The steers were on feed for an average of 273 days. At
slaughter, their lungs were evaluated for gross and histologic lesions indicative of pneumonia. Thirty-five percent of all steers were treated for respiratory disease at some point between birth and slaughter, but 72% had pulmonary lesions at slaughter. In those steers treated for respiratory disease, 78% were found to have pulmonary lesions, whereas 68% of the untreated steers also had pulmonary lesions. Regardless of treatment status, cattle with pulmonary lesions at slaughter had an ADG of .076 kg less than animals with no pulmonary lesions. Although it was apparent that treatment of respiratory disease did not prevent the formation of pulmonic lesions, it most likely reduced the severity of the pneumonia and ameliorated the potential production losses. It is also likely that extensive subclinical respiratory infections exist in most feedlots.

With the obvious economic significance of BRDC, numerous prevention and control measures have been evaluated for efficacy. Preconditioning of calves is one of these measures, wherein calves are vaccinated, dehorned, castrated, and weaned several weeks prior to transport to feedlots, in the hopes of reducing the stress these calves are subjected to after arrival. Unfortunately, this approach does not generally offer a pronounced financial benefit over treating BRDC as it develops, as most feedlot managers re-vaccinate these cattle, not trusting the seller’s claim of preconditioning. Another BRDC control strategy is mass medication of the feed or water supply during the transition from farm to feedlot. Due to the high costs and incomplete efficacy of mass medication in preventing BRDC, cost-benefit ratios indicate it is often more effective to treat cases of respiratory disease as they develop, rather than trying to medicate all animals.
Enhancing the immune system through the timely and logical use of vaccines is another attractive option for reducing the incidence and severity of BRDC. It has been found that a calf’s immune status early in life can affect its susceptibility to disease even after weaning, as calves diagnosed with failure of passive transfer (as measured by insufficient levels of IgG antibodies or plasma protein concentrations at 24 hours after birth) were more likely to suffer from respiratory disease both prior to weaning and in the feedlot. As vaccines to various pathogens thought to contribute to BRDC became available, they were often used indiscriminately, not allowing cattle enough time to develop a protective antibody response prior to challenge. Additional research has refined the use of many vaccines for maximum efficacy. Although many killed virus vaccines induced high levels of total antibodies, as was the case with bovine respiratory syncytial virus vaccine, the majority of these antibodies were non-neutralizing. The modified live vaccine resulted in higher levels of the needed neutralizing antibodies.

Alternative routes of vaccine administration are also being explored, beyond the traditional intramuscular or subcutaneous routes. Inducing humoral immunity may not be adequate to protect mucosal surfaces such as the respiratory tract. Applying an antigen directly to the mucosal surface, as with an intranasal vaccine, may induce a more effective local immune response, which would better protect against infection. Unfortunately, logistic limitations often prevent the use of intranasal vaccines in large numbers of feedlot cattle. Oral vaccines are another possibility for enhancing the immune response of the respiratory tract to invading organisms, as inducing immunity in gut-associated lymphoid tissue stimulates immunity at other mucosal portals, including the respiratory tract. Calves given oral doses of Pasteurella hemolytica culture
supernatants were found to be resistant to a subsequent intrabronchial challenge with the same bacteria.  

Ongoing studies continue to investigate means of enhancing natural immune responses. Gamma-delta T lymphocytes are common on mucosal surfaces of cattle and exhibit cytotoxic properties similar to both NK and T cytotoxic lymphocytes, leading to speculation that they contribute significantly to the immunological defense of mucosal surfaces. Recombinant cytokines may also offer a new way to improve immunity. Reduced viral shedding following BHV-1 challenge was seen in calves which had been given recombinant bovine interleukin-1 beta or recombinant bovine interleukin-2 and modified live BHV-1 virus, in addition to increased serum neutralizing antibody titers. Further research is needed to determine the practicality of incorporating such findings into morbidity-reducing feedlot protocols.

1.10 The Potential Role of BCV in BRDC

Considering the economic losses caused by BRDC, it is only reasonable to keep searching for additional ways to prevent and control it. As several other viruses have been implicated in the causation of BRDC, allowing vaccination against them to reduce their adverse effects, it is possible that BCV may also contribute to this disease complex. It has been isolated from outbreaks of calfhood pneumonia, 20,45,75 it has been propagated in cell and organ cultures derived from the bovine respiratory tract, 76,77 and it has been isolated from the respiratory tracts of experimentally infected calves. 80,81 With the ability to replicate in the epithelial cells of the upper respiratory tract, it is possible that BCV causes tissue damage and may compromise the host's defense mechanisms, allowing viruses and bacteria to colonize the lower respiratory tract. BCV may also act
synergistically with other infectious agents to cause more severe pulmonic lesions than any one agent acting alone.

Evidence exists to suggest BCV may contribute to BRDC in feedlot cattle. A report from Austria cited BCV as the cause of 2 outbreaks of bovine respiratory disease. It was isolated from sick calves up to 13 weeks of age, and from cattle without overt signs of respiratory disease up to 17 months of age. In a 1996 study conducted by Storz et al., nasal swabs from 100 cattle arriving at feedlots in Kansas and Arizona, showing clinical signs of respiratory disease (coughing, dyspnea, nasal discharge, and elevated rectal temperatures), were analyzed for the presence of BCV using cell culture inoculation. Respiratory BCV (BRCV) were isolated from 38 of the 100 cattle sampled, with the classification as BCV based on morphologic features, HA and receptor-destroying enzyme activities, cell fusion, and their cytopathic features in cell culture. PI3 viruses were also found in 4 of the cattle, but no other known bovine respiratory pathogens were found. The authors hypothesized that BCV infections in feedlot cattle were previously unrecognized due to either a lack of a permissive cell culture system, or that BCV is currently emerging as a pathogen in cattle populations due to the immune pressure of successful vaccination programs against other agents.

In a pilot study conducted in 1995, we surveyed 489 mixed-breed steers and heifers entering 4 Ohio feedlots in 5 groups for BCV nasal shedding and seroresponses. Nasal swabs were collected from all cattle at arrival, and serum samples were collected from 185 calves at arrival and again at 28 days on feed. Records were kept on treatment for clinical respiratory disease while the calves were on feed. Nasal swab supernatants were tested for the presence of BCV using a pool of 3 monoclonal
antibodies to BCV structural proteins in an antigen capture ELISA. The paired sera were tested for IgG antibodies to BCV using an antibody detection ELISA. We detected BCV antigen in 21.5% of calves, with prevalences of 3.3% to 56.2% within individual groups. Calves with positive antigen ELISA results were 2.5 to 3.2 times more likely to need treatment for clinical respiratory disease than those which were negative for BCV on ELISA. Tests of paired sera demonstrated seroresponses (defined as a four-fold or greater increase in titer) in 38.9% of the calves. These findings suggest that some cattle are shedding BCV as they arrive at feedlots, and that cattle maintained in feedlots develop seroresponses to BCV. An association between BCV shedding and clinical respiratory disease was also suggested.

Evidence continues to accumulate implicating BCV in BRDC. In 1998 Storz et al. cultured nasal swabs from 105 cattle involved in an outbreak of respiratory disease at a Texas feedlot. Inoculation of the samples into the G clone of human rectal tumor cells (HRT-18) revealed that 64 of the cattle were infected with BRCV at the order-buyer barn, and another 23 became infected prior to arrival at the feedlot. Nine of the 10 cattle that died during the outbreak were found to have BRCV infections, and 69 of the surviving 95 cattle were found to be infected with BRCV but no other viruses.

Another 1998 study evaluated the association between antibody titers to BCV and the occurrence of respiratory disease. Serum samples from 604 calves, fed in feedlots in Ontario and Alberta, were taken at arrival and at day 28 on feed. The samples were analyzed for BCV antibodies using virus neutralization tests, which were also used to detect antibodies to M. bovis, M. dispar, IBR, BRSV, PI3, and BRDV. The investigators found that 83% of the calves had detectable BCV antibody titers on arrival,
indicating exposure to BCV prior to arrival at the feedlot. The BCV titers of calves in Alberta were almost twice as high as those of the calves fed in Ontario, and the Alberta cattle had more respiratory disease than the Ontario calves, most likely owing to geographic differences in infection rates. Seroconversion (a 4-fold or greater increase in BCV titer) occurred in 61% to 100% of the calves in each group. Calves with titers to BCV at arrival appeared to be protected against BRD for the first month on feed, with each titer unit decreasing the risk of BRD by an odds ratio of 0.8. No relationship was found between seroconversion and risk of BRD, although titer change was strongly negatively correlated with the arrival titer. The relationship between arrival titer and BRD risk remained consistent even after the addition of titers to other pathogens to the statistical model. The investigators also found that higher BCV antibody titers promoted higher weight gains, most likely due to the observed protective effect against BRDC.

If BCV is found to contribute to BRDC, a protective vaccine could be developed to minimize its contribution. As with other respiratory pathogens, it is likely that local, intranasal administration of the BCV antigen would stimulate a better mucosal immune response than systemic vaccination. Due to the ability of mucosa-associated lymphoid tissue to cross-immunize, and the antigenic similarities between BRCV and BECV strains, it is also likely that oral administration of a BECV vaccine would also stimulate an immune response in the lymphoid tissue of the respiratory tract, as has been shown to happen in cross-protection studies of TGEV and PRCV.

Earlier research has defined the role of BCV in enteric disease and demonstrated its tropism for the bovine respiratory tract, but it has not adequately explored the
possibility of BCV contributing to the development of BRDC. Even after years of research and enormous progress being made in understanding the causes of BRDC, no generally effective program exists to protect feedlot cattle from the damage inflicted by the recognized components of BRDC. Thus, the search must continue for previously unrecognized agents and factors which exacerbate the effects of the more well-known causes of BRDC. BCV is likely one of these component causes. Understanding the epidemiology and pathogenicity of BRCV infections in feedlot cattle is the first step in developing prevention and control measures which may reduce the incidence and severity of BRDC, thus helping to reduce feedlot morbidity and mortality.

1.1 References


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CHAPTER 2

ADAPTATION OF AN ANTIGEN-CAPTURE ELISA TO DETECT BOVINE RESPIRATORY CORONAVIRUS IN NASAL SWABS

2.1 Summary

Bovine coronavirus is recognized as an important cause of neonatal calf diarrhea, and also contributes to winter dysentery in adult dairy cattle. Recently, investigators have isolated bovine coronavirus from feedlot cattle, but the epidemiology of this virus in feedlot populations and its potential contribution to the respiratory disease complex of feedlot cattle remains unknown. In order to survey large numbers of feedlot cattle for the presence of BCV, an antigen-capture ELISA originally developed for the detection of BCV in fecal samples from adult dairy cattle was adapted for rapid, reliable testing of nasal swabs from feedlot cattle. Three cell-culture adapted strains of BCV were used to test the analytical sensitivity of the ELISA, and the sensitivity, specificity, and efficiency of the ELISA were calculated using nasal swab samples from challenge-exposed calves. Using a pool of 3 monoclonal antibodies resulted in an ELISA with a sensitivity and specificity of 93.3%, with specimens collected from experimentally infected calves. A higher cutoff point was used for field samples, to increase specificity and reduce the number of false-positive samples. The described protocol can be used to rapidly screen
nasal swabs from large numbers of feedlot cattle, allowing the description of feedlot BCV epidemiology.

2.2 Introduction

Bovine coronavirus (BCV) has long been recognized as a cause of potentially fatal neonatal calf diarrhea, as well as being implicated in winter dysentery of adult dairy cattle. Recently, several studies have indicated that BCV may also contribute to the respiratory disease complex of feedlot cattle. Known to have a tropism for the bovine respiratory tract, and previously isolated from outbreaks of calfhood pneumonia, this single-stranded, positive-sense RNA virus has also been isolated from older cattle suffering from respiratory disease as they enter feedlots. Although cattle shedding the virus have been found to be at increased risk for clinical respiratory disease, it is still unknown if BCV is contributing to the development of respiratory disease, working synergistically with other viruses, bacteria, and environmental stressors, or if it is an incidental finding, as it can also be isolated from healthy cattle. Extensive surveys of feedlot cattle are needed to describe the epidemiology of respiratory BCV (BRCV), and to determine its potential pathogenicity. In order to assay the large numbers of samples required for such studies, a rapid, sensitive assay for BRCV was needed.

Methods of detection for enteric BCV (BECV) in feces include electron microscopy (EM), immunoelectron microscopy (IEM), and immunofluorescence (IF). Although these techniques are adequately sensitive for detecting virus in feces, the time and equipment required to perform them on large numbers of samples is prohibitive. Several investigators have developed enzyme-linked immunosorbent assays (ELISAs) to more rapidly detect the presence of BECV in fecal samples. Monoclonal antibodies
(MAbs), rather than polyclonal serum, are preferred for antigen capture, due to the improved sensitivity and specificity of ELISAs which incorporate MAbs.\textsuperscript{15,16}

Smith and colleagues\textsuperscript{16} developed an indirect double antibody antigen-capture ELISA for BECV using MAbs, in order to analyze large numbers of fecal samples from cattle diagnosed with winter dysentery (WD). The specificity of this ELISA was 100\% (95\% confidence interval of 88.3—100\%), and the sensitivity was 97.2\% (83.8--99.9\%).\textsuperscript{16} Additionally, the results from this ELISA were in agreement with EM/IEM results, yielding a Kappa value of 0.96 (.89-1.0)\textsuperscript{16} This assay was well-suited for detecting BECV in fecal samples, but needed to be adapted for the detection of BRCV in nasal swabs from feedlot cattle.

Shortly after adapting the Smith ELISA for detection of BRCV, as described in this chapter, da Silva et al. published a protocol for detection of respiratory coronaviruses using an ELISA.\textsuperscript{17} Although this ELISA demonstrated excellent specificity (95.4\%), its sensitivity was lower than desired (76.5\%).\textsuperscript{17} Additionally, this ELISA used only one MAb, directed against the S glycoprotein. It was therefore possible that the single MAb used would not react with all strains of BRCV, contributing to the lower level of sensitivity of the assay. For this reason, a pool of 3 MAbs, each to a different epitope, was used in the development of our ELISA, in an effort to improve sensitivity.

Although the exact antigenic relationship between BECV and BRCV strains continues to be investigated, evidence indicates they are similar in their antigenic and biologic properties.\textsuperscript{18,19} Reynolds et al. found that gnotobiotic calves inoculated with BECV strains were resistant to infection with certain BRCV isolates.\textsuperscript{18} Tsunemitsu et al.\textsuperscript{19} and Hasoksuz et al.\textsuperscript{20} studying the antigenic relationships of BCV strains isolated from
cases of calf diarrhea, winter dysentery, and pneumonia, found that some antigenic diversity exists among BCV strains, but the diversity appeared to be unrelated to the clinical source of the isolate.

In light of the apparent similarities between BECV and BRCV strains, it was decided to use the existing BECV ELISA as the basis for the BRCV ELISA, taking into consideration that nasal swabs were likely to have fewer viral particles present. Additionally, the conjugate used by Smith et al. was no longer available, necessitating its replacement in the assay and the appropriate adjustment of the other reagents, to maintain sensitivity and specificity. The antigen-capture ELISA described is suitable for testing large numbers of nasal swabs from feedlot cattle, and was used in a 3-year survey of feedlot cattle for the presence of BRCV.

2.3 Materials and Methods

Production of Monoclonal Antibodies: The MAbs available in sufficient quantity to use in the BRCV ELISA had previously been produced against the DB2 strain of calf diarrhea (CD) BECV, and the WD strains BM and DBA. No BRCV isolates were available at the time for production of MAbs, but anti-BRCV MAbs are currently being developed for future testing. Female Balb/c mice, 13 weeks old, were injected intraperitoneally with 0.1 ml of purified, inactivated BCV (80 µg of protein/mouse). Supernatants from the hybridomas were screened for antibody production using IF, fluorescent focus neutralization (FFN), and hemagglutination inhibition (HI) tests against homologous virus strains. Cloned cells from hybridomas, which were found to be producing antibodies to BCV on FFN and HI, were injected into pristane-primed mice to produce ascites. Five to 10 days after injection with 0.2 ml of hybridoma cells
(1x10^6 cells/ml), the ascites were collected. The isotypes of the MAbs produced were determined using an immunodiffusion assay with monospecific anti-mouse immunoglobulin sera. Antibody titers of the MAbs to BCV were determined using indirect immunofluorescence.

Testing of Monoclonal Antibodies: Six BCV MAbs were available in sufficient quantity to both standardize the test, and screen the large number of feedlot samples. Table 2.1 lists these MAbs, their protein specificities, and FA titers. Each MAb was tested individually in the existing ELISA, using a cell-culture adapted BRCV strain (BRCV 440), then grouped into pools. Previous studies demonstrated that pooling MAbs of varying protein specificities allowed them to compensate for one another in the case of epitope differences between BCV strains. These pools of 3 MAbs each were compared using three low-passage, cell-culture adapted BRCV strains from feedlot cattle (Figure 1), as well as a calf diarrhea BCV reference strain (Mebus) and a winter dysentery strain (DBA). The TCID_{50}/ml of BCV in cell culture medium was calculated using the Reed Muench method. Serial dilutions of these 5 strains were tested in the optimized ELISA to determine which pool would give higher analytical sensitivity.

Test Antigens: Initially, no BRCV isolates were available for testing, requiring the use of fecal samples from gnotobiotic or colostrum-deprived calves as the test controls. Six IEM BCV-positive samples and 6 IEM BCV-negative fecal samples, diluted 1:25 in phosphate buffered saline (PBS), were used to compare conjugates. After isolation of BRCV strains
(BRCV 440, 255, and 228) in human rectal tumor cells (HRT-18), dilutions of these strains were used to test candidate reagent combinations for analytical sensitivity.

**Virus Isolation:** In order to provide BRCV strains for both standardizing the ELISA and antigenic and biologic characterization, monolayers of HRT-18 cell cultures, grown in 6-well plates, were used for virus isolation, as described previously. After 72 hours of growth, HRT-18 cells were washed 3 times with cell culture medium (Eagles Minimal Essential medium (MEM), to which had been added 1% antibiotics (penicillin, mycostatin, and dihydrostreptomycin) and 1% NaHCO₃). The cells were inoculated with 0.1 ml of nasal swab supernatants which had been found to be ELISA-positive (using the previously established BECV ELISA). During the 1 hour adsorption, the plates were rotated every 15 minutes, then MEM with 5 µg/ml of pancreatin was added. The cultures were incubated at 37°C in a 5% CO₂ atmosphere, and harvested when 80% of the cells demonstrated cytopathic effects (CPE). Viruses were cloned by liquid limiting dilution, as the BRCV isolates did not consistently form plaques. Therefore, the highest dilution of virus which resulted in detectable CPE was passaged 3 times in HRT-18 cells. Aliquots of the cell culture supernatants were stored at −70°C until needed.

**Secondary Antibody:** Hyperimmune serum was previously prepared by injecting guinea pig #8609B with purified Mebus BCV mixed with Freund’s complete adjuvant. Five intramuscular injections of 0.5 ml each were administered 14 days apart, then the serum was collected 10 weeks after the initial inoculation and tested using virus neutralization (VN) for antibodies to BCV. The serum had a VN titer of 10,000 (the reciprocal of the highest serum dilution that inhibited 50% of the CPE).
**Conjugates:** Four different conjugates were tested, all of which were anti-guinea pig labeled with horseradish peroxidase. Affinity-purified anti-guinea pig antibodies produced in rabbits and goats, produced by Sigma, were tested, as well as a Boehringer Mannheim rabbit anti-guinea pig conjugate and an affinity-purified goat-anti guinea pig conjugate from Kirkegaard-Perry Laboratories.

**Checkerboard Testing:** To narrow the choices for optimal reagent combinations and concentrations, varying concentrations were tested on the same plate, to keep conditions as uniform as possible. Fecal samples known to be either IEM positive or negative were first tested with each candidate protocol, and the absorbances calculated by subtracting the average of the 2 negative-coated wells from the average of the 2 positive-coated wells. The results were then analyzed to determine sensitivity and specificity, and to maximize the signal for positive-coated wells and minimize non-specific reactions which would increase the background signal in negative-coated wells.

**Immunofluorescence Assays:** As nasal swabs taken from either challenge-exposed calves or field cases could not be tested using IEM, due to the low numbers of virus present and their fragility, nasal epithelial cells were tested for the presence of BCV using immunofluorescent (IF) microscopy. Swabs from both nostrils were placed in 4 ml of cell culture medium. After vortexing the samples, removing the swabs, and centrifuging the tubes at 110xg for 15 minutes to pellet the cells, the supernatants were removed and stored at -70°C until needed. The pelleted cells were washed and rinsed twice with PBS, then mixed and applied in triplicate (200 μl/well) to wells of HCT slides. After
air-drying, the cells were fixed with 100% acetone for 10 minutes at room temperature. The cells were rinsed with PBS (pH 7.2) and dried, then incubated at 37°C for 1 hour in a humid chamber with fluorescein isothiocyanate (FITC)-conjugated hyperimmune antiserum to the DB2 strain of BCV (diluted 1:10, 50 µl per well) on one well, FITC-conjugated hyperimmune serum to group A rotavirus (used at 1:100) on a second well, and PBS on a third well. After rinsing off the serum, 0.2 ml of PBS (pH 8.0) was added to each well and incubated for 10 minutes at room temperature. Using 30 µl of PBS (pH 8.0):glycerol (4:6) as a mounting solution, coverslips were applied and the cells examined for immunofluorescence, with the results reported as the percentage of cells positive for BCV (number of fluorescing cells out of 100 cells counted). This information was used to analyze the results of candidate ELISA protocols.

**ELISA Procedure:** For all tests, Nunc Maxisorp 96-well flat-bottom microtiter plates were used. The positive, paired rows were coated with pooled monoclonal antibodies to BCV in a carbonate-bicarbonate buffer (pH 9.6). Negative, paired rows were coated with ascites negative for BCV antibodies, at the same concentration as the MAbs in the positive rows, in the same buffer. The coating was applied at 100 µl per well, then the plates were sealed and incubated overnight at 4°C. Following incubation, the plates were washed using an ICN Titertek Plus M96 automated plate washer and PBS with 0.05% Tween 20, and blocked for 2 hours at room temperature with 200 µl per well of 5% non-fat dried milk to reduce non-specific binding. The plates were washed 5 times, then the samples applied, at 100 µl per well, for one hour at room temperature. After another 5 rinses, 100 µl of the secondary antibody, hyperimmune guinea pig serum to BCV, was
applied to the wells for one hour at room temperature. The plates were washed and incubated for an hour with a horseradish-peroxidase conjugated anti-guinea pig antibody. To develop color at the end of the assay, 100 μl of a 1:1000 solution of H₂O₂ and 2.2'-azino-bis(3-ethyl-benzthiazoline) sulfonic acid in 0.1 M sodium citrate was applied to each well for 20 minutes. The color development was stopped by adding 50 μl of 5% sodium dodecyl sulfate to each well. The plates were read at a wavelength of 414 nm on a computer-linked ELISA reader, and the optical densities saved as ASCII files. A Quattro Pro spreadsheet program was used to calculate the ELISA values for the samples, by subtracting the average absorbance of the paired negative-coated wells from the average absorbance of the paired positive-coated wells. The sensitivity, specificity, and efficiency (the mathematical product of sensitivity X specificity) of the assay were calculated using known IF-positive and IF-negative nasal specimens from colostrum-deprived and gnotobiotic calves experimentally challenged with BCV.

2.4 Results

Prior to optimizing the other reagents, a new anti-guinea pig peroxidase-labeled antibody had to be selected, as the previously used conjugate was no longer available. From the data presented in Figure 2.1, it became apparent that the K.PL goat anti-guinea pig conjugate minimized the background due to non-specific binding, while yielding the maximum positive signal, resulting in the largest difference in average absorbance values for positive and negative samples. Additional checkerboard tests were performed to determine the optimum concentration of MAbs for coating, blocking reagent, secondary antibody, and conjugate. Incubation times of 30, 60, and 120 minutes were tried for each step, and incubation at room temperature and at 37 C were investigated. Initial testing
indicated that using a 1:8000 dilution of MAbs, incubated on Nunc Maxisorp plates overnight at 4°C, a 2-hour room-temperature incubation with 5% non-fat dried milk, application of samples for an hour, followed by a 1-hour incubation with the hyperimmune secondary antibody diluted 1:2000, then application for 1 hour of KPL goat anti-guinea pig peroxidase-labeled antibody at 1:8000, with color development using ABTS for 20 minutes, yielded the most desirable results of any of the combinations tested. All incubations were performed at room temperature, as incubation at 37°C did not appear to affect the strength of the signal, or the time needed for signal development.

Using this protocol, 2 separate pools of MAbs (Table 2.1) were tested to determine the analytical sensitivity of the ELISA. All 5 strains of BCV were detected (Figures 2.2 and 2.3). The analytical sensitivities of each pool of MAbs, listed as minimum TCID₅₀/ml detectable for each BCV strain, are listed in Table 2.2. Pool 1 was selected for use in the BRCV ELISA, due to its greater analytical sensitivity when testing respiratory strains, and the increased magnitude of the signal for positive samples. Each MAb in the pool was used at a concentration of 1:8000.

Testing 30 IF-positive nasal swabs and 30 IF-negative nasal swabs from gnotobiotic and colostrum-deprived calves in the ELISA described above yielded the frequency distribution shown in Figure 2.4, with 28 of the 30 IF-negative nasal samples resulting in ELISA values of 0.02 or less, and 28 of the 30 IF-positive nasal samples resulting in ELISA values of greater than 0.02. To determine the optimum cutoff value for the ELISA, sensitivity, specificity, and efficiency were calculated across a range of cutoff values, from 0.01 to 0.1. Figure 2.5 illustrates the variation in sensitivity and specificity of the ELISA based on the selected cutoff value. In order to maximize
sensitivity, specificity, and efficiency, a cutoff of greater than or equal to 0.02 was selected for calf samples, resulting in a sensitivity of 93.3%, a specificity of 93.3%, and an efficiency of 87%. However, a cutoff of 0.1 was used for field samples from older cattle (>6 months), to reduce the number of false-positive results caused by non-specific reactions. In field samples, BRCV was isolated in cell culture only from samples with a corrected absorbance of 0.1 or greater. Although a higher cutoff for these samples increased the risk of false-negative results, the greater specificity (100%) of the results was judged to be worth the decrease in sensitivity (63%).

2.5 Discussion

Several ELISAs for the detection of BCV in fecal samples, both from calves and adult cattle, have been developed which demonstrate excellent sensitivity and specificity. These ELISAs use monoclonal antibodies to improve the specificity of the assay, as they can be directed against a single epitope of the virus. It can be difficult to detect specific viruses in fecal samples, due to the presence of multiple microorganisms, cellular debris, coproantibodies, and assorted biologic remnants. ELISAs used to detect BCV in fecal samples must be both specific and sensitive, in order to distinguish BCV from other enteric organisms. The previously developed BCV ELISAs were carefully optimized to detect BCV in fecal samples. However, an ELISA was now needed to detect relatively low amounts of virus in nasal swab fluids of feedlot cattle, in order to survey feedlot cattle for the presence of BCV. As the exact antigenic relationship between feedlot BRCV strains and previously-isolated BECV strains was unknown at the time of the development of the assay, further testing of, and possibly changes to, the existing BCV ELISA were needed to determine the appropriate protocol for detecting BRCV.
Detecting BCV in nasal swabs presents several challenges. As the virus infects the epithelial cells of the upper respiratory tract, swabs must be taken that contain infected cells. It is likely that small amounts of virus are present in the swabs, compared to fecal shedding. Additionally, BCV is sensitive to heat and light. In feces, the virus can be protected by surrounding fecal matter, but in nasal swabs it is more likely to be exposed to heat and light, further reducing the number of intact viral particles present in a given sample. Although the presence of BCV could be confirmed in fecal samples using EM and IEM, these techniques could not be reliably applied to respiratory samples, due to the low number of viral particles present, their fragility, and the difficulty of applying EM or IEM to large numbers of samples.

Additional drawbacks at the time of the development of the ELISA were the lack of controls known to be positive for BRCV, and the unavailability of the sheep anti-guinea pig antibody used as a conjugate in a previously developed BCV antigen-capture ELISA. An appropriate replacement, a goat anti-guinea pig conjugate manufactured by KPL, was found by initially using known IEM-positive and -negative fecal samples. By the time a conjugate was selected, cell culture passages of BRCV strains were available for further testing and modification of the ELISA, resulting in the protocol described in the results section.

The described protocol uses MAbs directed against the DB2 strain of calf diarrhea BECV, as a pool of 3 of these MAbs, directed against 3 of the 4 major structural proteins of BCV, was found to result in the most sensitive assay (Table 2.2). Recent investigations in our laboratory of field isolates of BRCV, and their comparison to previously isolated winter dysentery and calf diarrhea BECV strains, have found that antigenic similarities
exist between BECV and BRCV strains. Nine out of 10 BRCV strains, isolated from feedlot cattle, had properties similar to BECV subtypes when tested using VN and HI assays. One strain, BRCV 220, was not closely related to either BRCV or BECV strains, indicating some antigenic diversity exists, but that diversity cannot be ascribed solely to the clinical source of the virus. Although the anti-BECV MAbs used in this ELISA worked well, the sensitivity of the test may be improved from its current 93.3% (in experimentally challenged calves) and 63% (in field samples) by using a pool of the anti-BRCV MAbs which are currently being developed and tested.

By calculating the sensitivity, specificity, and efficiency of the ELISA at varying cutoff values, it was found that using a cutoff of 0.02 would prove the most efficient for samples from experimentally infected calves, reducing the number of false positives and false negatives. As the specificity and sensitivity are 93.3%, a small number of samples will be misclassified, as is the case with almost all diagnostic techniques. Additionally, basing the classification of the nasal samples on the IF results assumes that these results are infallible, which is not the case. However, with not being able to test the samples using IEM, the IF assay yielded the most reliable indication of the presence of BCV. A higher cutoff for field samples (0.1) decreased the sensitivity (63%) but resulted in a specificity of 100%, an acceptable alternative to increased numbers of false-positive samples.

The ELISA described by da Silva et al. had a specificity of 95.4% and a sensitivity of 76.5%, when compared to cell culture isolation. The ELISA described in this chapter has a higher sensitivity (93.3%), most likely due to the use of a pool of 3 monoclonal antibodies. Rather than using a single MAb, directed against the S
glycoprotein of BCV, as da Silva did, this ELISA incorporated one MAb directed against the S protein, one against the HE protein, and one against the N protein, allowing detection of a larger number of BRCV strains.

Recent studies have indicated BCV can be isolated in cell culture from feedlot cattle showing clinical signs of respiratory disease. However, no attempt was made to isolate the virus from healthy feedlot cattle, and the role of BCV in feedlot respiratory disease remained undefined. In order to define the epidemiology of BCV in feedlot cattle, and its potential contribution to the respiratory disease complex of feedlot cattle, large numbers of cattle, both healthy and ill, need to be sampled for the presence of BCV. The antigen-capture ELISA described can be used to rapidly screen large numbers of nasal swab samples from feedlot cattle, allowing the investigation of the role of BCV in feedlot respiratory disease.

2.6 Sources and Manufacturers

*a* Rabbit anti-guinea pig-IgG-POD Sigma, St. Louis, MO.

*b* Goat anti-guinea pig-IgG-POD Sigma, St. Louis, MO.

*c* Sheep anti-guinea pig-IgG-POD Boehringer Mannheim, Corp., Indianapolis, IN

*d* Goat anti-guinea pig-IgG-POD Kirkegaard & Perry Laboratories

*e* HCT Autoclavable 8-well slides Erie Scientific, Erie, PA.

*f* Nunc Maxisorp; Nunc, Inc., Naperville, IL.

*g* Titer Tek Plus M96 plate washer, Flow Laboratories Inc., McLean, VA.

*h* Titer Tek Multiscan plate reader, Flow Laboratories Inc., McLean, VA.

*i* Quattro Pro Windows v 7.0, Borland International Inc., Scotts Valley, CA.
2.7 References


<table>
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<tr>
<th>Hybridoma</th>
<th>Protein Specificity</th>
<th>Mebus</th>
<th>DBA</th>
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<tr>
<td><strong>MAb Pool 1</strong></td>
<td></td>
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<tr>
<td>BC 22 F8.3C*</td>
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<td>25,600</td>
<td>25,600</td>
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<td>BC 28 H1.2C</td>
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<td>102,400</td>
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<tr>
<td>BC 29 G7.2C</td>
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<td></td>
<td></td>
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<tr>
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<td>12,800</td>
</tr>
<tr>
<td>BM 02 D10 D7.F10</td>
<td>S</td>
<td>1,000</td>
<td>1,000</td>
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<tr>
<td>DBA 02 H6 B7.A10</td>
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<td>1,838,400</td>
<td>1,838,400</td>
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**Table 2.1**: Monoclonal antibodies to BCV tested for use in an antigen-capture ELISA

*Directed against the DB2 strain of BCV*
<table>
<thead>
<tr>
<th>BCV Strain</th>
<th>MAb Pool 1</th>
<th>MAb Pool 2</th>
</tr>
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<tbody>
<tr>
<td>BRCV 255</td>
<td>$10^{4.1}$</td>
<td>$10^{4.9}$</td>
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<tr>
<td>BRCV 440</td>
<td>$10^{3.7}$</td>
<td>$10^{5.2}$</td>
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<tr>
<td>BRCV 228</td>
<td>$10^{3.6}$</td>
<td>$10^{5.7}$</td>
</tr>
<tr>
<td>BECV DBA</td>
<td>$10^{3.3}$</td>
<td>$10^{4.0}$</td>
</tr>
<tr>
<td>BECV Mebus</td>
<td>$10^{4.1}$</td>
<td>$10^{3.3}$</td>
</tr>
</tbody>
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Table 2.2 Analytical sensitivities, listed as minimum TCID$_{50}$/ml detectable, for 2 pools of monoclonal antibodies (MAbs) used in a BCV antigen-capture ELISA.
Figure 2.1 Comparison of different conjugates in an antigen capture ELISA for BCV. Six IEM positive samples and 6 IEM negative samples were tested repeatedly at varying concentrations of each of the conjugates. The difference between the absorbances for the positive samples and negative samples was calculated at each concentration, in order to maximize the difference.
Figure 2.2 The end point of virus detection by ELISA using 3 BRCV strains and 2 pools of monoclonal antibodies. ELISA values greater than 0.01 were considered positive.
Figure 2.3 The end point of virus detection using 2 BECV strains and 2 pools of monoclonal antibodies. ELISA values >0.01 were considered positive.
Figure 2.4 Frequency distribution of ELISA values obtained by BCV antigen-capture ELISA from 30 FA-positive and 30 FA-negative reference nasal samples.
Figure 2.5 Sensitivity, specificity, and efficiency of BCV antigen-capture ELISA, calculated to determine the optimum cutoff value.
CHAPTER 3
DEVELOPMENT OF AN ELISA TO DETECT ANTIBODIES TO BOVINE RESPIRATORY CORONAVIRUS IN FEEDLOT CATTLE

3.1 Summary

Although bovine coronavirus has previously been acknowledged as a cause of enteric disease in both calves and adult cattle, its role in respiratory disease of older cattle remains unclear. Coronavirus has recently been isolated from outbreaks of respiratory disease in feedlot cattle, leading to questions about its possible contribution to the financially significant bovine respiratory disease complex of feedlot cattle. Little is known about the seroprevalence of BCV antibodies in feedlot cattle. In conjunction with surveys of feedlot cattle for nasal shedding of BCV, serological screening was also needed to further define the epidemiology of BCV in feedlots. A reliable BCV antibody detection ELISA was needed to screen hundreds of acute and convalescent samples from feedlot cattle. Using serum samples with known virus-neutralization BCV antibody titers, an antibody-detection ELISA was developed to detect BCV IgG antibodies in samples from feedlot cattle, which were found to have high levels of non-specific antibody binding. The assay developed reduced this background as much as possible, and allowed the rest to be removed mathematically. Defining seroconversion as a four-fold or greater increase in titer, the correlation between seroresponse, as measured by ELISA, and that
measured by VN, was good ($r^2=0.68, p<0.005$). The assay has subsequently been used to test over 800 samples from Ohio and Texas feedlot cattle.

3.2 Introduction

Bovine coronavirus (BCV) is an enveloped, single-stranded, positive-sense RNA virus which has long been recognized as a cause of potentially fatal neonatal calf diarrhea. $^{1,2}$ Epidemiologic studies have also implicated BCV in winter dysentery of adult dairy cattle. $^{3,4,5,6}$ In 1982, Thomas et al. first isolated BCV from lung washes and nasopharyngeal swabs from calves involved in 2 outbreaks of pneumonia. $^7$ Further investigations revealed the virus’ tropism for tissue and cell cultures derived from the bovine respiratory tract, $^{8,9}$ and resulted in the isolation of BCV from numerous cases of calfhood pneumonia. $^{10,11}$ As BCV is capable of infecting the bovine respiratory tract, $^{8,9}$ and is associated with calfhood pneumonia, $^{10,11}$ recent investigations have been designed to determine if BCV could also be associated with respiratory disease in older cattle, specifically the respiratory disease complex of feedlot cattle.

Records maintained by the National Animal Health Monitoring System (NAHMS) indicate that the annual loss of cattle to respiratory disease is higher than that for any other cause of death, with an estimated annual loss of $1$ billion. $^{12}$ Feedlot cattle are especially susceptible to respiratory disease, due to the additive effects of multiple infectious agents and environmental stressors the cattle are subjected to as they travel from the farm to the feedlot. $^{13,14,15}$ In a 1996 review of records from central United States feedlots, Edwards found that 67 to 82% of the total morbidity in feedlots stemmed from respiratory disease. $^{16}$ Economic losses are significant, resulting not only from death losses and treatment costs, but from preventive measures, additional personnel costs, and
reduced production in affected cattle. A Texas study\(^{17}\) found that cattle with clinical signs of respiratory disease during the feeding period gained 7% less weight, cost 18.5% more to feed, and weighed 3% less when shipped to packers than cattle which had not been diagnosed with respiratory disease during the feeding period. A large number of cases of respiratory illness may also go undiagnosed, as Wittum et al.\(^{18}\) found that 68% of steers in a Colorado feedlot which were never treated for clinical respiratory disease had pulmonary lesions when examined at slaughter.

Multiple infectious agents\(^{19,20,21,22}\) and physical stresses\(^{13,14,15}\) have been implicated in the pathogenesis of bovine respiratory disease complex (BRDC). The generally accepted theory of causation is that the innate immunologic defense mechanisms of the respiratory tract are overwhelmed by a combination of viral infections,\(^{19,20,21,22}\) including bovine respiratory syncytial virus (BRSV), bovine viral diarrhea virus (BVDV), bovine herpesvirus-1 (BHV-1), and parainfluenza-3 (PI3), and stresses, including transportation, inclement weather, changes in ration, and mixing with cattle from different sources.\(^{13,14,15}\) With the normal immune response suppressed, normal bacterial inhabitants of the upper respiratory tract, including *Pasteurella hemolytica* and *P. multocida*, can infect the lower respiratory tract and cause fibrinous bronchopneumonia, leading to illness, production losses, and death.

As other viruses have been implicated as contributing to the immunosuppression and pneumopathogenicity key to the development of BRDC, it is possible that BCV may also contribute to this development, given its tropism for the bovine respiratory tract and its role in calfhood pneumonia. Recently, investigators have isolated BCV from feedlot cattle as they arrived at feedlots.\(^{23,24}\) A 1996 study by Storz et al.\(^{23}\) found BRCV in 38
of 100 cattle arriving at feedlots in Arizona and Kansas and showing signs of clinical respiratory disease (coughing, nasal discharge, dyspnea, and elevated rectal temperatures). In a 1995 study conducted in 4 Ohio feedlots, we found nasal shedding of BCV in 21.5% of the calves tested. Calves shedding BCV at arrival were 2.5 to 3.2 times more likely to need treatment for clinical respiratory disease than those calves not shedding the virus. Evidence suggests BRCV may play a role in feedlot respiratory disease, but its epidemiology and possible role in causation remain unclear.

One of the problems faced in defining the epidemiology of BRCV in feedlot cattle is the transient shedding of the virus. In screening feedlot cattle for the virus, we found it to be shed primarily within the first 3 days after arrival. Although cattle can be sampled on arrival, as they are being processed, it is difficult to obtain samples again on day 1, 2 or 3 after arrival. Due to the operating procedures of most feedlots, cattle remain in their home pens to reduce stress for the first week on feed. Additionally, BCV is highly susceptible to heat and light, resulting in some virus being destroyed or nonviable in samples that must be transported long distances.

To further define the epidemiology of BRCV in feedlot cattle, a method of detecting exposure to the virus was needed, to add to the information obtained by testing nasal swabs for the presence of BRCV. Several BCV antibody detection ELISAs have been described for detection of enteric strains of BCV (BECV), including one developed for detection of BCV antibodies from adult cows with winter dysentery. A similar indirect antibody detection ELISA was adapted from Smith’s protocol to measure the IgG responses of feedlot cattle to BRCV during their first month on feed.
3.3 Materials and Methods

*Production of Monoclonal Antibodies:* The MAbs available in sufficient quantity to use in the BRCV ELISA had previously been produced against the DB2 strain of calf diarrhea (CD) BECV, and the WD strains BM and DBA. No BRCV isolates were available at the time for production of MAbs, but anti-BRCV MAbs have been developed for future testing. Female Balb/c mice, 13 weeks old, were injected intraperitoneally with 0.1 ml of purified, inactivated BCV (80 ug of protein/mouse). Supernatants from the hybridomas were screened for antibody production using IF, fluorescent focus neutralization (FFN), and hemagglutination inhibition (HI) tests against homologous virus strains. Cloned cells from hybridomas, which were found to be producing antibodies to BCV on FFN and HI, were injected into pristane-primed mice to produce ascites. Five to 10 days after injection with 0.2 ml of hybridoma cells (1x10⁶ cells/ml), the ascites were collected. The isotypes of the MAbs produced were determined using an immunodiffusion assay with monospecific anti-mouse immunoglobulin sera. Antibody titers of the MAbs to BCV were determined using indirect IF. A pool of 3 MAbs, one directed against the spike (S) protein of BCV (BC 29 G7.2C), one directed against the hemagglutinin esterase (HE) (BC 22 F8.3C), and one directed against the nucleocapsid (N) protein (BC 28 H1.2C), were used to capture the BCV antigen in this ELISA, as they had previously been shown to be effective in a BCV antigen-capture ELISA and in an ELISA to detect BCV antibodies from adult cows with winter dysentery. When a negative coating was needed, negative SP 2/0 myeloma cell ascites were used, at the same concentration in coating buffer as the MAbs.
Virus Isolation: In order to provide BRCV for both standardizing the ELISA and antigenic and biologic characterization, monolayers of HRT-18 cell cultures, grown in 6-well plates, were used for virus isolation, as described previously. After 72 hours of growth, HRT-18 cells were washed 3 times with cell culture medium [Eagles Minimal Essential medium (MEM)*], to which had been added 1% antibiotics (penicillin, mycostatin, and dihydrostreptomycin) and 1% NaHCO$_3$. The cells were inoculated with 0.1 ml of the seventh passage of BRCV strain 440, a field isolate of BRCV adapted to HRT-18 cells. Additional flasks were treated identically for preparation of mock-infected cells, except for the addition of 0.1 ml of cell culture medium rather than BRCV 440. During the 1 hour viral adsorption, the plates were rotated every 15 minutes, then MEM with 5 µg/ml of pancreatin$^b$ was added. The cultures were incubated at 37° C in a 5% CO$_2$ atmosphere, and harvested when 80% of the cells demonstrated cytopathic effects (CPE). Viruses were cloned by liquid limiting dilution, as the BRCV isolates would not consistently form plaques. Therefore, the highest dilution of virus which resulted in detectable CPE was passaged 3 times in HRT-18 cells. Flasks of infected HRT-18 cells were frozen at -70 C and thawed, which was repeated three times to release the virus from the cells. The contents of the flasks were pooled and centrifuged at 1000 xg for 30 minutes to remove cellular debris. Aliquots of the cell culture supernatants were stored at -70° C until needed.

Test Antigens and Sera: Table 3.1 lists the 5 BCV strains tested for possible use in the ELISA and their TCID$_{50}$/ml. Three of the strains (228, 255, and 440) were cell-culture adapted strains isolated from feedlot cattle, and two of the strains (DBA and Mebus)
were previously-isolated enteric BCV strains. Twenty-nine paired serum samples were selected from a population of cattle (located at a feedlot in Lucasville, OH in the fall of 1996) known to contain animals shedding BCV at arrival. Ten of these cattle were shedding BCV at arrival, and 19 were not. Blood had been drawn from these cattle via jugular venipuncture as they arrived at the feedlot, and again at 28 days on feed. Duplicate aliquots of serum were stored at -20 C until they could be tested. While virus neutralization tests were being performed on these selected serum samples, samples from adult dairy cattle with winter dysentery were used to test the candidate ELISA protocols. Once VN titers were known on a number of feedlot samples, these sera were used for testing. One pair of samples from a known-BCV negative experimental colostrum-deprived calf (exposed to Breda virus but not BCV) was also used in the testing, to serve as a negative control. The samples and their BCV VN titers are listed in Table 3.2, as well as their ELISA titers from the final protocol.

Conjugates: Three anti-bovine IgG antibodies labeled with horseradish-peroxidase were tested at concentrations of 1:2000, 1:4000, 1:8000, and 1:12,000. Two goat anti-bovine IgG conjugates from Kirkegaard & Perry Laboratories were tested, one directed against the heavy and light chains of the molecule, and one specific to the gamma chain, to reduce binding to IgM antibodies. A rabbit anti-bovine IgG (heavy and light chains) from ICN was also tested.
**Microtiter plates:** Four different types of 96-well flat-bottom rigid plates were tested for use in the BCV antibody detection ELISA: the Nunc Maxisorp, \(^{f}\) regular Nunc plates, \(^{g}\) Immulon 1B plates, \(^{h}\) and Falcon plates. \(^{i}\)

**ELISA Procedure:** Initially, the ELISA protocol developed by Smith et al.\(^{27}\) was used, with modifications made to accommodate differences in serum samples. Various 96-well flat-bottom microtiter plates were used. \(^{f,g,h,i}\) All rows were coated with pooled monoclonal antibodies in a carbonate-bicarbonate buffer (pH 9.6), at a concentration of 1:8000. \(^{27}\) The coating was applied at 100 µl per well, then the plates were sealed and incubated overnight at 4°C. Following incubation, the plates were washed using an ICN Titertek Plus M96 automated plate washer \(^{j}\) and PBS with .05% Tween 20, and blocked for 2 hours at room temperature with 200 µl per well of 5% non-fat dried milk \(^{k}\) to reduce non-specific binding. The plates were washed 5 times, then BCV antigen applied, (Table 3.1) at 100 µl per well, for one hour at room temperature to the positive rows. Below each row coated with antigen, mock-infected HRT-18 cell lysates in cell culture medium were applied at the same dilution as the antigen. After another 5 rinses, 100 µl of an initial 1:25 dilution and two-fold dilutions (in PBS) of each serum sample were applied.

Following one hour incubation, the plates were washed and incubated for an hour with an affinity-purified horseradish-peroxidase conjugated goat anti-bovine IgG antibody. The chromogenic enzyme substrate consisted of 100 µl of a 1:1000 solution of H_2O_2 and 2.2'-azino-bis(3-ethyl-benzthiazoline) sulfonic acid in 0.1 M sodium citrate, applied to each well for 20 minutes. The color development was stopped by adding 50 µl of 5% sodium dodecyl sulfate to each well. The plates were read at a wavelength of 405 nm on a
computer-linked ELISA reader, and the optical densities saved as ASCII files. An Excel spreadsheet program was used to calculate the ELISA values for the samples, by subtracting the absorbance of the mock-coated wells from the absorbance of the BCV antigen-coated wells. The titer was determined to be the serum dilution at which the positive well had an OD of 0.1 greater than that of the negative well. Repeated testing of standards with known VN titers found this value consistently gave results similar in trend to those of the VN results.

3.4 Results

Initial attempts to use an existing BCV antibody-detection ELISA, developed for detecting BCV IgG antibodies in the serum of experimentally challenged calves and adult dairy cattle diagnosed with winter dysentery, were frustrated by the high levels of background in the feedlot field serum samples. Initially using Nunc Maxisorp plates, different antigens (Table 3.1), conjugates, blocking agents, and reagent concentrations were tried. Field samples of dairy cows with known serum neutralization antibody titers to BCV (Table 3.2) were initially used to screen the candidate protocols, as the presence of antibodies to multiple pathogens would more closely mimic the immune status of feedlot cattle, rather than using serum from gnotobiotic or colostrum-deprived calves. One paired serum sample from an experimental calf exposed to rotavirus and known to be negative for BCV antibody, was used to check the specificity of the tests. As the VN titers of feedlot samples became known, several of these paired samples were also tested in the various protocols (Table 3.2)

Preliminary testing of the 5 semipurified strains of BCV (see Chapter 2) and their testing in candidate ELISA protocols revealed that BRCV strain 440 was the preferred
antigen to use in testing for antibodies to BRCV. The analytical sensitivity of the Pool 1 MAbs used in the ELISA was $1 \times 10^{3.6}$ TCID$_{50}$/ml for BRCV 440. The isolate used had a TCID$_{50}$/ml of $1 \times 10^{8}$. Additionally, antigenic and biologic characterization of BRCV strains $^{32}$ found BRCV 440 to be both representative of most BRCV strains, yet similar enough to BECV strains to detect antibodies to them. Pools of BRCV 440 were grown in HRT-18 cells to provide enough antigen for screening ELISA protocols and testing the large numbers of paired field samples.

When testing combinations of reagents, results were compared to VN titers to check for similarities in trend. Although VN and ELISA tests will not yield identical results, as antibodies detected by ELISA may not necessarily be neutralizing antibodies, general increases or decreases in titers should be similar.$^{33}$ In addition to detecting seroconversion or its absence in paired samples, another objective of the ELISA testing was to reduce background, which tended to be high in the feedlot samples. Many of the protocols resulted in unacceptably high levels of background, with antibodies binding non-specifically directly to the plate. Several types of microtiter plates, with lower binding affinities than the Nunc Maxisorp plates originally used, were tested and found to yield better results for testing feedlot samples. The preferred plate, which gave strong signals for positive samples while also yielding background absorbances less than those of the other types tried, was the Dynex Immulon IB.

Using a lower-binding affinity microtiter plate helped reduce the background, but levels were still higher than desired. Several blocking agents at various concentrations were compared, including fish skin gelatin and non-fat dried milk (NFDM). Blocking
with 5% NFDM for two hours at room temperature proved most effective, dropping background levels in wells with no serum to an acceptable level of 0.09 or less.

Non-specific binding of antibodies in the field samples still occurred, however, and had to be accounted for in the layout of the plates. Coating alternate rows of each plate with coating buffer containing negative ascites, at the same concentration as the monoclonal antibodies being used, did not adequately reduce the non-specific binding. The preferred solution was to coat all rows overnight with a 1:8000 dilution of MAbs, then apply mock-infected HRT-18 cell lysates to alternating rows of each plate. Thus each serum sample was applied to a row to which BRCV antigen had been applied, to provide a positive absorbance, and to a negative row coated with mock-infected cell lysates, rather than antigen. The absorbances of the negative-coated rows were subtracted from the absorbances of the positive rows at each dilution of the serum. The remaining absorbance was that due to BRCV-specific binding.

When testing various possible reagent combinations, the absorbance values for positive wells were plotted across the serial serum dilutions, as were the absorbances for negative wells, as well as the difference between these two values and their ratio (an example is given in Figure 3.1). This allowed comparisons of candidate protocols based on the magnitude of differences in absorbance of serum samples with known BCV titers.

Seroconversion was defined as a four-fold or greater increase in titer from the acute sample to the convalescent sample, taken 28 to 30 days after the acute sample. Figure 3.2 plots the log 2 change in VN titer against the log 2 change in ELISA titer for 29 paired feedlot serum samples. Calculating the kappa statistic for agreement between these two tests resulted in a value of 0.58 (Table 3.3), a value which indicates a fair to
good agreement beyond that due to chance. The correlation coefficient for the changes in titer was 0.68 (p<0.05), also indicating an acceptable level of agreement between the two tests.

A pair of serum samples used in evaluating the ELISA protocols was tested periodically throughout the 4 months during which the feedlot samples were being tested, to analyze the consistency of the test. Background levels were monitored by leaving the last column free of serum. Alterations in background or changes in the standard resulted in re-calibration of the test and repetition of suspect samples.

3.5 Discussion

ELISAs have long been used to rapidly and accurately analyze large numbers of serum samples, providing a more rapid method than serum neutralization tests. Utilizing the sensitivity of enzymes and monoclonal antibodies, ELISAs can be used to quantitate levels of specific antibody isotypes, as well as differentiating antibodies to closely related strains of virus. In our current study, a BCV antibody-detection ELISA was needed to screen large numbers of acute and convalescent serum samples from feedlot cattle, in order to better understand the epidemiology of BRCV infections in feedlot cattle.

In developing the assay, the primary problem faced was the high level of non-specific binding, particularly in convalescent samples. Antibodies would bind directly to the microtiter plates, making it difficult to isolate BCV-specific IgG antibodies. It is not uncommon for feedlot cattle to develop large-magnitude antibody responses to a number of infectious agents during their first month on feed. Not only are the cattle vaccinated against many pathogens during this time, they are also exposed to other agents via mixing
with cattle from multiple sources. At no other point in their lives will they be exposed to as many agents in as short a period of time.

To improve the specificity of antibody binding, lower affinity plates and blocking with NFDM were used, as well as mathematically removing the effect of non-specific binding. Using a strain of BRCV, 440, previously found to be broadly reactive with most BCV strains ensured that most BCV antibodies would be detected. The comparison of ELISA results to VN results was favorable, as an exact duplication of titer magnitudes would not be expected. The VN test measures only neutralizing antibodies, whereas an ELISA will detect even non-neutralizing antibodies. When defining seroconversion as a four-fold or greater increase in titer, both diagnostic tests were in good agreement for the feedlot samples tested. The ELISA was subsequently used to evaluate the presence of IgG antibodies to BCV in large numbers of serum samples from cattle in feedlots in Ohio and Texas.

A recent study of 604 calves in Canadian feedlots found 82% of the calves had antibody titers to BCV on arrival at the feedlot. Seroconversion to BCV, as defined by a four-fold or greater increase in BCV neutralization antibody titer, within the first month on feed occurred in 61% to 100% of these calves, and it appeared that calves with a BCV antibody titer on arrival were protected against respiratory disease for the first month on feed. There was, however, no linear or multivariable association between BCV titer change and respiratory disease, or BCV titer and weight gain. The authors hypothesize this may be due to the strong correlation between BCV arrival titer and respiratory disease occurrence, and mention the need for further studies of the effects of BCV on bovine respiratory disease.
In our 1995 pilot study, \textsuperscript{24} seroconversion to BCV was found in 38.5% of the cattle tested. Evidence indicates that BCV may act as a typical feedlot respiratory pathogen, with a few cattle shedding the virus at arrival. Through mixing of cattle and immunosuppression due to stress and concurrent infections, most of the cattle have been exposed to BCV by the end of the first month on feed, and have developed an immune response to it. However, the exact role of BCV in respiratory disease in feedlot cattle remained unclear, and additional surveys of feedlots were conducted to better define the epidemiology and potential significance of BCV. The BCV antibody detection ELISA described allowed the testing of large numbers of samples, in order to present a clearer picture of the prevalence and infection patterns of BCV in feedlot cattle.

3.6 Sources and Manufacturers

\textsuperscript{a}Minimum Essential Medium, GibcoBRL, Grand Island, NY.

\textsuperscript{b}Pancreatin, GibcoBRL, Grand Island, NY.

\textsuperscript{c}Goat anti-bovine-IgG (H & L), Kirkegaard & Perry Laboratories, Gaithersburg, MD.

\textsuperscript{d}Goat anti-bovine-IgG (gamma), Kirkegaard & Perry Laboratories, Gaithersburg, MD.

\textsuperscript{e}Rabbit anti-bovine IgG (H & L), ICN, Costa Mesa, CA.

\textsuperscript{f}Nunc Maxisorp Microtiter Plate, Nunc, Inc., Naperville, IL.

\textsuperscript{g}Nunc Microtiter Plate, Nunc, Inc., Naperville, IL.

\textsuperscript{h}Immulon IB, Dynex Technologies, Chantilly, VA.

\textsuperscript{i}Falcon Microtiter Plates, Becton Dickinson Labware, Franklin Lakes, NJ.

\textsuperscript{j}Titertek Plus M96 plate washer, Flow Laboratories Inc., McLean, VA.

\textsuperscript{k}Non-fat dried milk, Scot Lad Foods Inc., Pewaukee, WI.
3.7 References


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<th>BCV Strain</th>
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<td>Mebus</td>
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<tr>
<td>DBA</td>
<td>1 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
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<td>BRCV 228</td>
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<tr>
<td>BRCV 255</td>
<td>1 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>BRCV 440</td>
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Table 3.1 BCV strains tested in BCV antibody-detection ELISA.
<table>
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<tr>
<th>Sample Number</th>
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<th>VN Titer</th>
<th>ELISA Titer</th>
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<td>Acute</td>
<td>2000</td>
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Table 3.2 VN and final ELISA titers of serum samples used in the testing of various BCV antibody-detection ELISAs. WD=winter dysentery samples, BO=experimental calf samples, negative for BCV antibodies, SF=feedlot samples.
<table>
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<td>BCV IgG antibody seroresponse</td>
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<td>21</td>
</tr>
<tr>
<td>ELISA</td>
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<td>1</td>
</tr>
</tbody>
</table>

Kappa=.58
$r^2=0.68$

**Table 3.3** Demonstration of agreement between VN seroresponses and ELISA responses for 29 paired serum samples from feedlot cattle.
Figure 3.1 Example of graphs used to analyze ELISA protocols. The ODs from negative, mock-antigen infected wells were subtracted from the ODs from positive, antigen-coated wells for a serum sample (WD 1022) with a known VN titer to BCV. In the second graph, the ratios of the positive well OD to that of the negative well were calculated. The selected protocol maximized the difference and ratio of the positive and negative wells at each dilution.
Figure 3.2 Scatter plot of the log 2 change in titer, as measured by virus neutralization (VN) tests versus the log 2 change in titer as measured by the BCV antibody detection ELISA. Seroconversion was defined as a change in titer of 2 or more (four-fold or greater increase in titer).
CHAPTER 4
THE DETECTION OF BOVINE RESPIRATORY CORONAVIRUS IN POPULATIONS OF FEEDLOT CATTLE

4.1 Summary

Objective—To describe the patterns of respiratory bovine coronavirus (BRCV) shedding and seroconversion in populations of feedlot cattle.

Design—Prospective observational study

Animals—1074 calves in Ohio, Texas, and Nebraska feedlots.

Procedure—Nasal swabs were taken at arrival and throughout the first month on feed, and tested for the presence of BRCV using an antigen-capture ELISA. Serum samples were collected at arrival and again at 28 days on feed and analyzed for antibodies to BRCV using an antibody-detection ELISA.

Results—Surveying 12 groups of cattle entering 7 different feedlots over 3 years, 7.3% of the cattle were found to be shedding BRCV, with a range of 0 to 35.9% for individual groups. 94% of the BRCV shedding occurring during the first week on feed. At arrival, 62.4% of the cattle had low (<50) BCV antibody ELISA titers. Seroconversion to BRCV (as defined by a four-fold or greater increase in BRCV antibody titer) was found in 58% of the cattle tested, with ranges for individual groups from 20.3% to 84.1%. Arrival titers to BRCV appeared to be negatively correlated with seroconversion to the virus during the first month on feed.
Conclusions—BRCV can be detected in varied populations of feedlot cattle, in the form of both viral shedding and seroconversion to the virus.

4.2 Introduction

Bovine coronavirus (BCV), a large, single-stranded, positive-sense RNA virus, has long been recognized as a significant cause of potentially fatal neonatal calf diarrhea.\(^1,2\) Investigators have also described its role in winter dysentery of adult dairy cattle.\(^3,4\) In 1982, Thomas et al.\(^5\) first isolated BCV from lung washes and nasopharyngeal swabs from calves involved in 2 outbreaks of pneumonia. Over the years, BCV has been isolated from numerous outbreaks of calfhood pneumonia,\(^5,6,7\) contributing to the theory that BCV may play a causative role in respiratory disease in cattle. Recently, investigators have also isolated BCV from outbreaks of respiratory disease in feedlot cattle.\(^8,9,10\) Cattle shedding the virus at arrival have been found to be at increased risk for respiratory disease,\(^9\) but it is still unknown if this is an incidental finding, as the virus can also be isolated from healthy cattle,\(^10\) or if respiratory BCV (BRCV) is contributing to the bovine respiratory disease complex (BRDC) of feedlot cattle.

The generally accepted theory of BRDC causation is that a combination of viruses and physical stresses overwhelm the normal immunological defense mechanisms of the respiratory tract, allowing normally commensal bacteria of the respiratory tract, including *Pasteurella hemolytica* and *P. multocida*, to infect the lower respiratory tract and cause fibrinous bronchopneumonia, leading to illness, production losses, and death. Numerous viruses have been implicated in the web of BRDC causation, including bovine respiratory syncytial virus (BRSV), and bovine herpesvirus-1 (BHV-1).\(^11,12,13,14\) These viruses, in combination with environmental stresses including transportation, mixing of
cattle from numerous sources, changes in ration, and inclement weather, increase the susceptibility of incoming cattle to bacteria which ordinarily would not be pathogenic.

Given the acknowledged roles of other viruses in BRDC, it is possible that BRCV may also act synergistically with other infectious agents and stresses to contribute to pneumonia in feedlot cattle. However, the prevalence of BRCV infections in feedlot populations and the rate of seroconversion to it are unknown. In order to define the epidemiology of feedlot BRCV infections, an observational study of 1074 mixed-breed cattle, entering 7 feedlots in 12 groups in Ohio, Texas, and Nebraska over a 3-year period (spring 1996 through fall 1998), was performed to obtain data on BRCV shedding patterns and rates of seroconversion to BRCV.

4.3 Materials and Methods

Study Populations: All cattle sampled were mixed-breed steers and heifers between 4 and 7 months of age. Three Ohio feedlots were used, one in the southern part of the state (Lucasville), one in the central part of the state (Jackson heifers, housed in Columbus), and one in the northeastern part of the state (Wooster). Cattle entering the Lucasville feedlot were purchased from a livestock auction in nearby Kentucky, originating from farms in Kentucky and Ohio. The Jackson cattle were raised on a branch farm of the Ohio Agricultural Research and Development Center (OARDC), with steers being fed at the OARDC Wooster feedlot and heifers at the Ohio State University feedlot in Columbus. In the fall of 1996, the Wooster cattle consisted of steers raised at 3 separate OARDC branch farms (Coshocton and Columbus, in addition to the Jackson steers), then mixed with cattle purchased at a Virginia livestock auction. In the fall of 1997, only the cattle
purchased at the livestock auction in Virginia, then transported to the Wooster feedlot, were sampled. The Texas cattle were obtained from various western Texas ranches, and mixed at a feedlot located near Amarillo. The fall 1998 samples came from Nebraska feedlots, and were obtained primarily in order to provide BRCV strains for antigenic comparison to strains isolated from Ohio cattle. In 8 of the groups of cattle, data were also available regarding treatment for respiratory disease and average daily gain. Cattle were treated according to each feedlot’s case definition and treatment protocol. Although the case definition of respiratory disease varied between feedlots, it generally consisted of an elevated rectal temperature, weight loss, inappetance, depression, coughing, nasal discharge, or any combination of these signs.

Sample Collection: At arrival and for varying days on feed (Table 4.1), nasal swabs were taken from the cattle. All cattle in an incoming group, both those suffering from respiratory disease and those that appeared to be healthy, were sampled. Using sterile, cotton-tipped applicators, both nostrils were swabbed, and the swabs placed in 4 ml of Eagles minimal essential medium*, to which had been added 1% antibiotics (penicillin, mycostatin, and dihydrostreptomycin) and 1% NaHCO3. The tubes were then vortexed, the swabs removed, and the tubes centrifuged at 1000x g for 11 minutes. The supernatants were removed, aliquoted into 2-2 ml portions, and stored at -70 C. In addition to nasal swabs, serum samples were obtained at arrival and again at 28 days on feed, in order to test for seroconversion to BRCV. Ten to 15 ml of whole blood were obtained via jugular venipuncture. The blood samples were centrifuged at 2000x g, then the serum was removed and aliquoted into duplicate portions and frozen at -20 C.
BRCV Antigen ELISA: An indirect, double-antibody sandwich antigen-capture ELISA previously developed by Smith et al.\textsuperscript{18} to detect BCV in fecal samples from adult dairy cattle was adapted to determine the presence of BCV in nasal swab supernatant fluids. Paired rows of Nunc Maxisorp\textsuperscript{a} 96-well microtiter plates were coated with either 100 µl /well of a 1:8000 dilution of a pool of 3 monoclonal antibodies (one to the BCV spike (S) protein, one to the hemagglutinin esterase (HE) protein, and one to the nucleocapsid (N) protein), or a 1:8000 dilution of BCV-antibody negative ascites (SP 2/0), in a carbonate-bicarbonate buffer of pH 9.6. Plates were incubated at 4 C overnight, then rinsed and blocked with 200 µl /well of 5% non-fat dried milk\textsuperscript{c} to reduce non-specific binding. After washing with a Titertek plate washer,\textsuperscript{d} 100 µl of nasal swab supernatant fluids were applied to 2 BCV antibody-positive coated wells and 2 BCV-antibody negative coated wells. The plates were incubated at room temperature for 1 hour, then rinsed. Guinea pig anti-BCV hyperimmune serum, at 1:2000, was applied to all wells at 100 µl /well for 1 hour at room temperature, followed by washing. A goat anti-guinea pig horseradish-peroxidase conjugated IgG antibody\textsuperscript{e} was applied at a dilution of 1:8000 for 1 hour. To develop color at the end of the assay, 100 µl of a 1:1000 solution of H\textsubscript{2}O\textsubscript{2} and 2,2'-azino-bis(3-ethyl-benzthiazoline) sulfonic acid in 0.1 M sodium citrate was applied to each well for 20 minutes. Color development was stopped by adding 50 µl of 5% sodium dodecyl sulfate to each well. The plates were read at 414 nm on a computer-linked ELISA reader\textsuperscript{f}, and the optical densities saved as ASCII files. A Quattro Pro spreadsheet program\textsuperscript{g} was used to calculate the ELISA values for the samples, by subtracting the average of the paired negative-coated wells from the average absorbance.
of the paired positive-coated wells. Samples with a resulting absorbance of 0.1 or greater were considered to be positive for BCV.

**BRCV Antibody ELISA:** An antibody-detection ELISA previously developed by Smith et al.\(^1\) for enteric BCV (BECV) was adapted to detect antibodies to BCV in serum samples from feedlot cattle. Adaptations to the previously described procedure\(^2\) included the addition of a blocking step (5% non-fat dried milk, applied for 2 hours at room temperature) to reduce the high levels of non-specific binding seen with the feedlot samples, as compared to the serum samples from adult dairy cattle used in the original test. Dynex Immulon 1B 96-well microtiter plates\(^3\) were used, rather than Nunc Maxisorp plates, to further reduce non-specific binding. Tissue culture supernatants of a BRCV strain (isolated from a calf in an Ohio feedlot and grown in HRT-18 cells) was used as antigen, rather than the enteric BCV strain DBA. An affinity-purified goat anti-bovine IgG antibody, conjugated to horseradish peroxidase\(^4\), was used for antibody detection, rather than the previously-described rabbit anti-bovine IgG. The E Max Precision Microplate Reader\(^5\) was used at a wavelength of 405 nm to determine the absorbances of each well. The absorbance from a row of wells coated with mock-infected cell culture supernatant, rather than BRCV antigen, was subtracted from the absorbance of the BRCV-coated wells at each dilution for each sample, using an Excel spreadsheet program.\(^6\) The titer was determined to be the serum dilution at which the positive well had an absorbance of 0.1 greater than that of the negative well.
**Virus Neutralization Assay:** A subset of 47 paired serum samples (5% of each group) was tested via virus neutralization (VN), as described previously, for comparison with ELISA results. A plaque reduction VN test was performed, using the BECV Mebus strain and HRT-18 cells. VN antibody titers were expressed as the reciprocal of the highest dilution of serum which resulted in an 80% reduction in plaque formation, as compared to a virus control.

### 4.4 Results

Table 4.1 lists the locations, dates, and numbers of cattle in each of the feedlot study groups. From the spring of 1996 through the fall of 1998, 12 groups of cattle entering 7 different feedlots were sampled, for a total of 1074 cattle. Serum samples, and nasal swabs taken after arrival, were available on 837 cattle (18 of the cattle in these groups died before the end of the feeding period, and convalescent serum samples were missing on 5 cattle). Sampling schedules (Table 4.1) varied between feedlots, depending on their handling procedures and any concurrent studies being performed. As an example, the cattle housed at the Lucasville feedlot were being sampled by another investigator for BRSV, necessitating frequent handling, whereas the Texas cattle were not processed prior to two weeks on feed.

Table 4.2 lists the number of animals shedding BCV from the upper respiratory tract, as detected by antigen-capture ELISA, for each of the days on feed listed. The overall number of animals shedding BRCV was relatively low, with 83 positive samples from 1074 cattle. Five of the positive samples were from animals previously found to be shedding the virus, giving an overall shedding rate of 7.3%. Examining the distribution of the numbers of positive animals, it becomes apparent that most shedding of the virus...
occurred during the first week on feed. Including all 12 groups of cattle, 78 out of 83 (94%) animals positive for BCV were found in the first week on feed. Excluding the 3 groups from the fall of 1998, as no additional samples were available from these animals, 68 of 73 (93%) positives occurred during the first week on feed. The percentage of positive samples per group varied widely, from 0 in the Jackson cattle in the fall of 1996, to 35.9% in the second lot of Lucasville during the same time frame.

Table 4.3 gives the numbers and percentages of animals in each herd that seroconverted to BRCV during the first month on feed. During the first 28 days on feed, 58% of the animals tested seroconverted to BCV on ELISA, with seroconversion being defined as a four-fold or greater increase in the BCV antibody titer. Only 20.3% of the Lot #2 calves housed at Lucasville in the spring of 1996 seroconverted to BCV, while almost 78% of the calves housed at the same location in the fall of 1996 seroconverted. Table 4.3 also provides the average acute and convalescent BCV antibody titers, as determined by ELISA, for each of the 9 groups for which this information was available. The average BCV titer at arrival, for the 814 cattle for which both acute and convalescent samples were available, was 404, while the average convalescent titer was 2242. Several groups demonstrated notable increases in average BCV titers during the first month on feed, including the cattle kept in Lucasville in the fall of 1996, and the Wooster, Jackson, and Texas cattle tested in the fall of 1996. Lot #2 of the spring Lucasville cattle demonstrated only a slight increase in average BCV over the first 28 days on feed.

Table 4.4 provides the numbers and percentages of cattle shedding BCV and seroconverting to it, based on their ELISA antibody titer to BCV at arrival. The majority of animals (62.4%) had low (less than 50) BCV antibody titers on arrival. The
percentages of animals which seroconverted to BCV during the first month on feed were similar in both the low-titer and high-titer groups, 82.5% and 87.6%, respectively. As seen from the data in Table 4.4, 72.1% of the cattle shedding BCV seroconverted, while only 63.3% of animals not shedding the virus seroconverted. The geometric mean acute and convalescent titers for the BCV-positive animals were lower than those for BCV-negative animals. Cattle not shedding BRCV were more likely to seroconvert to BCV than those shedding the virus (Table 4.5). However, in both groups of cattle, those with and without measurable BCV antibody titers at arrival, the majority of cattle seroconverted to BRCV. Table 4.6 provides a comparison between acute and convalescent BCV antibody titers and seroconversion rates for serum from 47 cattle tested using both VN assays and the antibody-detection ELISA. The correlation coefficient for the two test results was 0.56. The seroconversion rates for the 47 cattle are similar for both assays.

The distribution of acute and convalescent BCV antibody ELISA titers is given in Figure 4.1. Convalescent titers ranged from less than 50 to over 51,200. Figures 4.2 to 4.4 provide the distribution of acute and convalescent BCV antibody titers for each of the individual groups. Similar trends were seen when looking at the individual groups, with most animals having low BCV antibody titers at arrival, then a higher frequency of higher titers after the first month on feed. The groups of cattle fed at Lucasville during the spring of 1996 (Figure 4.2) had larger numbers of cattle with BCV antibody titers greater than 100 at arrival than the other groups of cattle. This fact is also apparent in the data in Table 4.3. The spring 1996 Lucasville cattle had the lowest rates of seroconversion to BCV and the highest BCV titers on arrival (Table 4.3).
Figure 4.5 demonstrates the percentages of cattle which seroconverted to BCV based on their arrival titers. The range of cattle which seroconverted among cattle arriving with low BRCV antibody titers (less than 200) was 67.3% to 75.8%. The percentage seroconverting dropped to less than 50% for cattle with arrival titers of at least 400 at arrival, and it dropped to 0 for arrival titers of 3200 and greater.

4.5 Discussion

The first step in determining the potential role of BRCV in the respiratory disease complex of feedlot cattle is to define the epidemiology of the virus in feedlot populations, including shedding patterns and exposure to the virus, as measured by seroconversion. Previous studies provided evidence BRCV could be isolated from feedlot cattle, but relatively small numbers of cattle were sampled in these studies. In 1996 Storz et al. isolated BCV from 38 of 100 cattle arriving at feedlots in Kansas and Arizona and showing signs of clinical respiratory disease. However, no healthy cattle were sampled in this study. In a 1995 study of cattle arriving at 4 Ohio feedlots, nasal shedding of BRCV was found in 21.5% of the calves tested. Calves shedding BCV at arrival were 2.5 to 3.2 times more likely to need treatment for respiratory disease than those calves not shedding the virus. Seroconversion to BCV, as defined by a four-fold or greater increase in BRCV antibody titer, occurred in 38.9% of the calves tested. In 1998 Storz et al. isolated BCV from 87 of 105 cattle involved in a respiratory disease outbreak at a Texas feedlot. Nine of the 10 cattle that died during the outbreak were found to have BCV infections, and 69 of the surviving 95 cattle were found to be infected with BCV but no other viruses.
In 1998 Martin et al. analyzed 604 paired serum samples, taken from calves at arrival at feedlots in Canada and again on day 28 on feed, for the presence of antibodies to BCV, using virus neutralization tests. It was found that 83% of the calves had detectable BCV antibody titers on arrival, indicating exposure to BCV prior to arrival at the feedlot. Seroconversion, as defined by a four-fold or greater increase in BCV antibody titer, occurred in 61% to 100% of the calves in each group.

Our results showed that BCV could be detected from cattle in Ohio, Texas, and Nebraska feedlots, at an overall rate of 7.3%. Rates of shedding of BCV from the upper respiratory tract varied between years and locations, as would be expected with any feedlot pathogen. No BCV was detected in 2 of the groups (Table 4.2), the second lot of spring, 1996 Lucasville cattle and the fall, 1996 Jackson cattle. Other groups also had very low rates of BCV shedding, including the Milford, NE calves and Texas calves. Two possibilities seem likely; either the virus was not common in those groups of cattle, or the cattle shed the virus between sampling dates. After arrival, the second highest number of BCV-positive samples was found on day 3 on feed (35 of 83 positive samples were found on day 3). In order to reduce stress on cattle following their arrival at the feedlot and assignment to home pens, most feedlots do not process cattle again prior to day 7 on feed. It is likely that more BCV-positive samples would have been found if more cattle had been available for sampling on day 3, rather than just the Lucasville cattle.

It is apparent from the data in Table 4.3 that the majority of cattle (58%) seroconverted to BCV during their first month on feed, with a range for individual groups from 20.3% to 77.9%. The higher average BRCV antibody titers at arrival apparently
reduced the percentage of spring 1996 Lucasville cattle which seroconverted, with rates of 20.3% to 49.3%, as compared to 53.1% to 84.1% in other groups. These cattle were purchased at a livestock auction, and were likely to have been previously exposed to the virus, allowing many to mount an immune response prior to arrival at the Lucasville feedlot. Although no BCV was found in the Jackson cattle, 84.1% of them seroconverted to the virus, as measured by ELISA. These cattle were mixed with calves from other branch farms, which may have been shedding the virus and serving as a source of exposure for the Jackson cattle.

It is apparent from the data in Tables 4.4 through 4.6 that over half of the cattle had low BCV ELISA antibody titers as they arrived at their feedlots. However, testing serum samples using a VN assay detected the presence of BCV antibodies in all acute samples tested, indicating that even those cattle with low BCV ELISA antibody titers (≤50) most likely did have a detectable, albeit low, level of BCV antibodies. This is similar to Martin's finding that over 80% of incoming cattle in the Canadian feedlots were seropositive for BCV when tested using VN assays. Although only a relatively few cattle were found to be actively shedding the virus, which could be due to shedding of the virus on days when cattle were not sampled, or shedding during holding and shipping following exposure to the virus at auction barns, by the end of the first month on feed most cattle had been exposed to the virus and developed a measurable seroresponse to it.

The distributions of acute and convalescent titers (Figures 4.1 to 4.4) were similar for most groups. Most cattle arrived with a relatively low titer to BCV, which then increased during the first month on feed. As noted previously, the spring, 1996 Lucasville
cattle tended to arrive with higher BCV antibody titers than animals in other groups, most likely due to previous exposure to the virus either at their farms of origin or, more likely, at the auction barn. Cattle with higher BCV antibody titers at arrival (Figure 4.6) were less likely to seroconvert, as they had most likely been previously exposed to the virus and thus would not produce the four-fold increase in antibody level needed to be classified as seroconversion. This finding is in agreement with that of Martin et al., who found that the change in BCV antibody titer during the first month on feed in Canadian feedlots was strongly negatively correlated with arrival titer.

The available BCV shedding and seroconversion data from this survey suggest that BRCV followed a pattern typical of other feedlot respiratory pathogens. A few cattle arriving at the feedlots were shedding the virus, most commonly during the first week on feed. These cattle were then mixed with previously unexposed cattle, serving as a source of exposure for BRCV. During the first month on feed, most cattle were exposed to the virus and developed a detectable antibody response to it. Cattle arriving with relatively high BRCV antibody titers were less likely to seroconvert than cattle without BRCV titers at arrival.

Similar patterns have been found with other respiratory pathogens in feedlot cattle. Extensive mixing of cattle from different origins at feedlots increases their risk for fatal fibrinous pneumonia, by exposing the calves to pathogens to which they have not been previously exposed. Evidence of seroconversion to numerous pathogens in feedlot cattle, during the first month on feed, has been documented by Martin et al. Seroconversion to P. hemolytica, PI3, and BRSV during the first month on feed was found in over 50% of Canadian feedlot calves sampled. Seroconversion to IBR was rare.
(<5%), but 40% of the calves seroconverted to Mycoplasma and BVDV during the first month on feed. Martin also found that arrival titers for these organisms were negatively correlated with subsequent seroconversion. In a 1992 study, Allen et al. found seroconversion to PI3 in 44 to 65% of cattle sampled, to BVD in 51%, to BRSV in 82 to 88%, and to P. hemolytica in 42 to 48% of feedlot cattle surveyed.

It is apparent that BCV can be isolated from feedlot cattle, in a variety of geographical locations, and that most cattle develop an antibody response to BCV after their first month on feed. However, further analysis is needed to determine the extent of damage caused by the presence of BCV and to more clearly define its contribution to BRDC. The presence of BCV in feedlot populations is incriminating, but does not, in and of itself, prove it causes respiratory disease.

4.6 Sources and Manufacturers

\textsuperscript{a}Minimal Essential Medium, GibcoBRL, Grand Island, NY.

\textsuperscript{b}Nunc Maxisorp Microtiter Plate, Nunc, Inc., Naperville, IL.

\textsuperscript{c}Nonfat dried milk, Scot Lad Foods, Inc., Pewaukee, WI.

\textsuperscript{d}Titertek Plus M96 plate washer, Flow Laboratories Inc., McLean, VA.

\textsuperscript{e}Goat anti-guinea pig-IgG-POD, Kirkegaard & Perry Laboratories, Gaithersburg, MD.

\textsuperscript{f}Titertek Multiscan plate reader, Flow Laboratories Inc., McLean, VA.

\textsuperscript{g}Quattro Pro Windows v. 7.0, Borland International Inc., Scotts Valley, CA.

\textsuperscript{h}Immulon 1B, Dynex Technologies, Chantilly, VA.

\textsuperscript{i}Emax Precision microplate reader, Molecular Devices, Sunnyvale, CA.

\textsuperscript{j}Microsoft Excel 97, Microsoft Corporation, Seattle, WA.
4.7 References


<table>
<thead>
<tr>
<th>Location</th>
<th>Arrival Date</th>
<th>Days on Feed Sampled</th>
<th>Number in Herd</th>
</tr>
</thead>
<tbody>
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<td>Lucasville</td>
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<td>0,3,6</td>
<td>69</td>
</tr>
<tr>
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<td></td>
<td></td>
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<td>0,3,6</td>
<td>70</td>
</tr>
<tr>
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<td></td>
</tr>
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<td>105</td>
</tr>
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<td></td>
<td></td>
</tr>
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</tr>
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<td></td>
</tr>
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</tr>
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<td>0,7,14,28</td>
<td>100</td>
</tr>
<tr>
<td>Jackson</td>
<td>10/11/96</td>
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<td>44</td>
</tr>
<tr>
<td>Texas</td>
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<td>0,14</td>
<td>109</td>
</tr>
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<td>Wooster</td>
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<tr>
<td>Hastings, NE</td>
<td>11/3/98</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Milford, NE</td>
<td>12/14/98</td>
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<td>50</td>
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<td><strong>Total</strong></td>
<td><strong>1074</strong></td>
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Table 4.1 Arrival dates for each group of cattle in BRCV survey, the days on feed on which nasal swabs were taken, and the total number of cattle in each group.
<table>
<thead>
<tr>
<th>Group</th>
<th>DOF 0</th>
<th>DOF 3</th>
<th>DOF 7</th>
<th>DOF 14</th>
<th>DOF 28</th>
<th>Total Positives</th>
<th>Percentage of Herd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lucasville Spr 96</td>
<td>0</td>
<td>7</td>
<td>0</td>
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<td></td>
<td>7</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lucasville Spr 96</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lucasville Spr 96</td>
<td>8</td>
<td>5</td>
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<td></td>
<td>13</td>
<td>12.4</td>
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</tr>
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<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>4</td>
<td>4.7</td>
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</tr>
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<td>8</td>
<td>23</td>
<td>1</td>
<td>1</td>
<td></td>
<td>33</td>
<td>35.9</td>
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</tr>
<tr>
<td>Wooster Fall 96</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>Jackson Fall 96</td>
<td>0</td>
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<td>0</td>
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<td>Texas Fall 96</td>
<td>2</td>
<td>1</td>
<td>1</td>
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<td>2.7</td>
</tr>
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<td>2</td>
<td>1</td>
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<td></td>
<td>12</td>
<td>7.4</td>
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<td>Scottsbluff, NE Fall 98</td>
<td>6</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td>6.9</td>
</tr>
<tr>
<td>Hastings, NE Fall 98</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>3.0</td>
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<tr>
<td>Milford, NE Fall 98</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2.0</td>
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<tr>
<td><strong>Total</strong></td>
<td>41</td>
<td>35</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>83</td>
<td>7.3</td>
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Table 4.2 Number of samples positive for BRCV on ELISA, grouped by days on feed, number of total positives per group, and the percentage of positive animals in each herd.
<table>
<thead>
<tr>
<th>Group</th>
<th>Number Seroconverting To BCV</th>
<th>Percent Seroconverting To BCV</th>
<th>Day 0 BCV GMT</th>
<th>Day 28 BCV GMT</th>
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</thead>
<tbody>
<tr>
<td>Lucasville Spr 96</td>
<td>34</td>
<td>49.3</td>
<td>365.4</td>
<td>1611.4</td>
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<td>Lot #1</td>
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<td></td>
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<td>Lucasville Spr 96</td>
<td>14</td>
<td>20.3</td>
<td>678</td>
<td>735.7</td>
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<td>Lot #2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lucasville Spr 96</td>
<td>46</td>
<td>43.8</td>
<td>1550.6</td>
<td>2249.2</td>
</tr>
<tr>
<td>Lot #3</td>
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<td></td>
<td></td>
</tr>
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<td>Lucasville Fall 96</td>
<td>67</td>
<td>77.9</td>
<td>160.2</td>
<td>4852.6</td>
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<td></td>
</tr>
<tr>
<td>Lucasville Fall 96</td>
<td>71</td>
<td>77.2</td>
<td>247</td>
<td>2405.3</td>
</tr>
<tr>
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<td></td>
</tr>
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<td>Wooster Fall 96</td>
<td>56</td>
<td>56</td>
<td>65.1</td>
<td>959.1</td>
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<td>Jackson Fall 96</td>
<td>37</td>
<td>84.1</td>
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<td>5525.9</td>
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<td>53.1</td>
<td>173.4</td>
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<tr>
<td>Total</td>
<td>473</td>
<td>58.0</td>
<td>404</td>
<td>2242</td>
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Table 4.3 Numbers and percentage of each group which seroconverted (a 4-fold or greater increase in ELISA titer) to BCV, and the geometric mean titers (GMT) to BCV at day 0 and day 28 on feed.
<table>
<thead>
<tr>
<th>BRCV Titer at Arrival</th>
<th>&lt;50</th>
<th>&gt;50</th>
<th>Percent Seroconverting</th>
<th>Acute GMT</th>
<th>Convalescent GMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (percent)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRCV +</td>
<td>54</td>
<td>14</td>
<td>72.1</td>
<td>84</td>
<td>1413</td>
</tr>
<tr>
<td>BRCV -</td>
<td>454</td>
<td>292</td>
<td>63.3</td>
<td>434</td>
<td>2318</td>
</tr>
<tr>
<td>Seroconverted</td>
<td>419</td>
<td>268</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Did not seroconvert</td>
<td>89</td>
<td>38</td>
<td></td>
<td></td>
<td></td>
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Table 4.4 BRCV shedding status, seroconversion rates, and geometric mean BRCV titers for arrival and 28 days on feed for 814 feedlot cattle.
<table>
<thead>
<tr>
<th>Arrival Titer</th>
<th>BRCV Status</th>
<th>Seroconversion (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>≤ 50</td>
<td>+</td>
<td>40 (74.1)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>379 (83.5)</td>
</tr>
<tr>
<td>&gt; 50</td>
<td>+</td>
<td>9 (64.3)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>259 (88.7)</td>
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Table 4.5 BRCV seroconversion rates for 814 feedlot cattle, classified according to BRCV arrival titer and BRCV shedding status.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Arrival GMT</th>
<th>Convalescent GMT</th>
<th>Seroconversion Rate</th>
</tr>
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<tr>
<td>VN</td>
<td>1018</td>
<td>10588</td>
<td>78.70%</td>
</tr>
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<td>ELISA</td>
<td>100</td>
<td>1884</td>
<td>72.30%</td>
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Table 4.6 Comparisons of acute and convalescent geometric mean BRCV titers and seroconversion rates for virus neutralization and ELISA results from 47 cattle.
Figure 4.1 Frequency distribution of ELISA titers to BCV in 814 feedlot cattle. Acute samples were taken at arrival, and convalescent samples were taken at 28 days on feed.
Figure 4.2 Frequency distributions of acute and convalescent BCV ELISA antibody titers for the 3 lots of cattle housed at Lucasville during the spring of 1996, and the 2 lots housed there in the fall of 1996.
Figure 4.3 Frequency distributions of acute and convalescent BCV ELISA antibody titers for cattle at the Wooster feedlot in the fall of 1996 and the fall of 1997.
Figure 4.4 Frequency distributions of acute and convalescent BCV ELISA titers for cattle from Jackson, OH and Amarillo, TX in the fall of 1996.
Figure 4.5 Percentage of animals seroconverting for each level of BRCV arrival titer.
5.1 Summary

Objective—To determine the relationship between bovine respiratory coronavirus (BRCV) infection and the incidence of treatment for respiratory disease and average daily gain.

Design—Prospective longitudinal

Animals—838 calves in feedlots in Ohio and Texas

Procedure—Nasal swabs were taken at arrival and throughout the first month on feed, and tested for the presence of BRCV using an antigen-capture ELISA. Serum samples were collected at arrival and again at 28 days on feed and analyzed for antibodies to BRCV using an antibody-detection ELISA. Information was collected on treatment for respiratory disease and average daily gain. Statistical analysis was used to determine the effects of BRCV shedding and seroconversion on these outcomes.

Results—BRCV shedding was found in 8.1% of the cattle tested. The majority of cattle tested (58%) seroconverted to BRCV during the first month on feed. A significant interaction was found between antigen shedding and seroconversion when assessing the risk for respiratory disease. Cattle shedding the virus and developing an antibody response to it were 1.6 times more likely to need treatment for respiratory disease than
those animals which neither shed the virus nor developed an immune response to it. No effect on average daily gain was seen.

Conclusions—Bovine respiratory coronavirus infects feedlot cattle and appears to increase their risk for respiratory disease. Developing appropriate control measures could help reduce the incidence of respiratory disease in some situations.

5.2 Introduction

Bovine coronavirus (BCV) was first recognized as a cause of potentially fatal neonatal calf diarrhea in 1972. Additional investigations found this large, enveloped, single-stranded RNA virus not only in outbreaks of calf diarrhea, and winter dysentery of adult dairy cattle, but in cases of calfhood pneumonia as well. Following Thomas' isolation of BCV from lung washes and nasopharyngeal swabs from calves involved in 2 outbreaks of pneumonia in 1982, further investigation confirmed the tropism of BCV for the bovine respiratory tract. Although these studies detected the virus in young calves (less than 6 months old), recent investigations have revealed that BCV can also be isolated from the respiratory tracts of older cattle. With the isolation of BCV from the respiratory tracts of cattle involved in respiratory disease outbreaks at feedlots, the question has arisen concerning the possible role BCV may play in the bovine respiratory disease complex (BRDC) of feedlot cattle.

Surveys of feedlots reveal respiratory disease to be one of the most financially significant causes of morbidity and mortality among feedlot cattle. Cattle arriving at feedlots are subjected to multiple stresses after leaving their farm of origin, including transportation over long distances, mixing with unfamiliar cattle, changes in ration, and processing, which can include vaccination, deworming, castration, and dehorning.
The mixing of cattle from multiple sources exposes them to previously-unencountered infectious agents, further increasing their susceptibility to illness. The combination of physical stresses and viral agents, including bovine herpesvirus-1 (BHV-1), infectious bovine rhinotracheitis (IBR), parainfluenza-3 (PI-3), and bovine respiratory syncytial virus (BRSV), leads to immunosuppression of normal defense mechanisms, and the colonization of the lower respiratory tract with normally commensal organisms of the upper respiratory tract, such as *Pasteurella multocida* and *P. hemolytica*.\(^{18,19,20,21}\) The pneumonia caused by these pathogens can result in significant production losses, treatment costs, and death losses.

Considering the roles of multiple viruses in the causation of BRDC, it seems likely that BCV, with its tropism for the respiratory tract and association with calfhood pneumonia, could also contribute to the pathogenesis of BRDC. In our 1995 pilot study, conducted in Ohio feedlots, we found BCV shedding in 21.5% of the cattle tested, and seroconversion (a four-fold or greater increase in BCV ELISA titer) to BCV in 38.9% of the cattle.\(^{22}\) Cattle shedding the virus were 2.5 to 3.2 times more likely to be treated for respiratory disease than those cattle not shedding the virus.\(^ {22}\) In 1996, Storz et al.\(^ {12}\) isolated BCV from 38 of 100 cattle involved in respiratory disease outbreaks at feedlots in Kansas and Arizona. In a 1998 study, Storz et al.\(^ {23}\) found BCV shedding in a Texas feedlot at even higher rates, with 87 of 105 cattle showing clinical signs of respiratory disease shedding the virus. Evidence of exposure to BCV has also been found in Canada, where Martin et al.\(^ {24}\) detected seroconversion to BCV in 51% to 100% of the calves tested, with high BCV titers at arrival appearing to provide protection against the development of respiratory disease. However, as no virus isolation from the respiratory
tract was attempted, it is not known if the BCV antibodies resulted from exposure to an enteric strain, or a respiratory strain, of BCV.

Although evidence of BRCV infections in feedlots is available, it is still unclear what role it may play in BRDC, as we found that the virus could also be isolated from apparently healthy cattle. In order to better define the role of BRCV in BRDC, we surveyed 838 mixed-breed cattle, entering 3 feedlots in Ohio and one in Texas, over a two-year period (spring of 1996 through the fall of 1997), in order to describe BRCV shedding patterns and rates of exposure. Information was collected on treatment rates for respiratory disease and average daily gain for these cattle, to allow statistical modeling of the effects of BRCV shedding and seroconversion on these outcomes. This information, in addition to experimental challenge of calves with field isolates of BRCV, will help clarify the role of BRCV in feedlot respiratory disease.

5.3 Materials and Methods

Study Populations: All cattle sampled were between 4 and 7 months of age, and were predominantly mixed-breed. Both steers and heifers were sampled. Three Ohio feedlots were used, one in the southern part of the state (Lucasville), one in the central part of the state (Jackson heifers, housed in Columbus), and one in the northeastern part of the state (Wooster). Cattle entering the Lucasville feedlot were purchased from a livestock auction in nearby Kentucky, originating from farms in Kentucky and Ohio. The Jackson cattle were raised on a branch farm of the Ohio Agricultural Research and Development Center (OARDC), with steers being fed at the OARDC Wooster feedlot and heifers at the Ohio State University feedlot in Columbus. In the fall of 1996, the Wooster cattle consisted of steers raised at 3 separate OARDC branch farms (Coshocton and Columbus,
in addition to the Jackson steers), then mixed with cattle purchased at a Virginia livestock auction. In the fall of 1997, only the cattle purchased at the livestock auction in Virginia, then transported to the Wooster feedlot, were sampled. The Texas cattle were obtained from various western Texas ranches, and mixed at a feedlot located near Amarillo. All cattle were vaccinated at arrival at their respective feedlots, with multivalent vaccines including vaccines against IBR, BVD, and PI3 viruses. Information was collected on treatment for respiratory disease and average daily gain. Cattle were treated according to each feedlot's case definition and treatment protocol. Although the case definition of respiratory disease varied between feedlots, it generally consisted of an elevated rectal temperature, weight loss, inappetance, depression, coughing, nasal discharge, or any combination of these signs.

Sample Collection: At arrival and for varying days on feed, nasal swabs were taken from the cattle. All cattle in an arriving group, both those suffering from respiratory disease and those that appeared to be healthy, were sampled. Using sterile, cotton-tipped applicators, both nostrils were swabbed, and the swabs placed in 4 ml of Eagles minimal essential medium, to which had been added 1% antibiotics (penicillin, mycostatin, and dihydrostreptomycin) and 1% NaHCO₃. The tubes were then vortexed, the swabs removed, and the tubes centrifuged at 1000x g for 11 minutes. The supernatants were removed, aliquoted into 2-2 ml portions, and stored at -70 C. In addition to nasal swabs, serum samples were obtained at arrival and again at 28 days on feed, in order to test for seroconversion. Ten to 15 ml of whole blood were obtained via jugular venipuncture. The
blood samples were centrifuged at 2000x g, then the serum was separated and aliquoted into duplicate portions and frozen at -20 C.

**BRCV Antigen ELISA**: An indirect, double-antibody sandwich antigen-capture ELISA previously developed by Smith et al.⁵ to detect BCV in fecal samples from adult dairy cattle was adapted to determine the presence of BRCV in nasal swab supernatant fluids. Paired rows of Nunc Maxisorp ⁶ 96-well microtiter plates were coated with either 100 µl/well of a 1:8000 dilution of a pool of 3 monoclonal antibodies (one to the BCV spike (S) protein, one to the hemagglutinin esterase (HE) protein, and one to the nucleocapsid (N) protein), or a 1:8000 dilution of BCV antibody negative serum, in a carbonate-bicarbonate buffer of pH 9.6. Plates were incubated at 4 C overnight, then rinsed and blocked with 200 µl/well of 5% non-fat dried milk ⁷ to reduce non-specific binding. After washing with a Titertek plate washer, ³ 100 µl of nasal swab supernatant fluids were applied to 2 positive-coated wells and 2 negative-coated wells. The plates were incubated at room temperature for 1 hour, then rinsed. Guinea pig anti-BCV hyperimmune serum, at 1:2000, was applied to all wells at 100 µl/well for 1 hour at room temperature, followed by washing. A goat anti-guinea pig horseradish-peroxidase conjugated IgG antibody ⁸ was applied at a dilution of 1:8000 for 1 hour. To develop color at the end of the assay, 100 µl of a 1:1000 solution of H₂O₂ and 2,2'-azino-bis(3-ethyl-benzthiazoline) sulfonic acid in 0.1 M sodium citrate was applied to each well for 20 minutes. Color development was stopped by adding 50 ml of 5% sodium dodecyl sulfate to each well. The plates were read at 414 nm on a computer-linked ELISA reader, and the optical densities saved as ASCII files. A Quattro Pro spreadsheet program was used.
to calculate the ELISA values for the samples, by subtracting the average of the paired negative-coated wells from the average absorbance of the paired positive-coated wells. Samples with a resulting absorbance of 0.1 or greater were considered to be positive for BRCV.

*BRCV Antibody ELISA:* An antibody-detection ELISA previously developed by Smith et al. for enteric BCV (BECV) was adapted to detect antibodies to BRCV in serum samples from feedlot cattle. Adaptations to the previously described procedure included the addition of a blocking step (5% non-fat dried milk, applied for 2 hours at room temperature) to reduce the high levels of non-specific binding seen with the feedlot samples, as compared to the serum samples from adult dairy cattle used in the original test. Dynex Immulon 1B 96-well microtiter plates were used, rather than Nunc Maxisorp plates, to further reduce non-specific binding. Tissue culture supernatant of a BRCV strain (isolated from a feedlot in Ohio and grown in HRT-18 cells) was used as antigen, rather than the BECV strain DBA. An affinity-purified goat anti-bovine IgG antibody, conjugated to horseradish peroxidase, was used for antibody detection, rather than the previously-described rabbit anti-bovine IgG. A different plate reader, the E Max Precision Microplate Reader, was used at a wavelength of 405 nm to read the plates. The absorbance from a row of wells coated with mock-infected cell culture supernatant, rather than BRCV antigen, was subtracted from the absorbance from the BRCV-coated wells at each dilution for each sample, using an Excel spreadsheet program. The titer was determined to be the serum dilution at which the positive well had an absorbance of 0.1 greater than that of the negative well.
Testing for Additional Pathogens: Subsets of the samples were analyzed for the presence of infection with other known respiratory viruses. Serum samples from the fall 1996 Texas cattle were tested, using virus neutralization tests, for antibodies to IBR, BVDV, PI3, and BRSV. Nasal swab supernatants from the cattle housed at Lucasville in the fall of 1996 were tested, using cell culture virus isolation, for the presence of IBR, BVDV, and BRSV.

Statistical Analysis: The outcomes of interest in the analysis were treatment for respiratory disease at any time during the feeding period and average daily gain during the feeding period. The primary independent variables of interest were the shedding of BRCV and seroconversion to BRCV during the first month on feed. The acute BRCV titer was also included, to account for varying levels of immunity to BRCV at arrival. Group, gender, and entry weight were tested for potential confounding. The effects of these variables on the dichotomous outcome of treatment for respiratory disease were assessed using multivariable logistic regression. Multivariable linear regression was used to assess the effects of the independent variables on the continuous outcome of average daily gain. The initial model contained BRCV shedding status and BRCV seroconversion. The effects of potentially confounding variables, including gender and weight at entry, were assessed using forward selection with a criteria of \( p < 0.05 \) for inclusion in the model. The interaction between BRCV shedding and seroconversion was tested for using the same selection criteria. For the Texas cattle, the effects of acute titers and seroconversion to IBR, BVDV, BRSV, and PI3 were tested in both models.
5.4 Results

Of the cattle surveyed, 8.1% were shedding BRCV (Table 5.1), with ranges for individual groups from 0 to 35.9%. Five of the positive samples were from cattle with one other positive sample, thus 68 BRCV-positive cattle were represented by the 73 positive nasal swabs. Shedding was most likely to occur during the first 7 days on feed, with 94% of the positive samples detected during this time. Over 53% of the cattle tested were treated for respiratory disease during the feeding period, with ranges from 0 to 72.5%. Whereas 52.5% of the cattle which did not shed BRCV were treated for respiratory disease, 64.7% of cattle shedding BRCV from the upper respiratory tract were treated for respiratory disease. None of the Jackson cattle, one of only 2 groups of cattle in which no BRCV was found, were treated for respiratory disease. The Texas cattle, with a BRCV shedding rate of only 2.7%, also had a relatively low treatment rate, of only 24%. In all other groups at least 40% of the cattle were treated for respiratory disease, with a range of 43.5% to 72.5%. During the first 28 days on feed, 58% of the animals tested seroconverted to BRCV by ELISA, with seroconversion being defined as a fourfold or greater increase in titer. Seroconversion rates for individual groups ranged from 20.3% to 84.1%.

When testing for the effects of BRCV shedding and seroconversion on treatment for respiratory disease, gender and entry weight did not appear to confound the effects, given that group effects were controlled for by including the group number. Table 5.2 lists the parameters included in the final model for predicting treatment of respiratory disease. Gender, entry weight, and shedding of BRCV during the first 3 days on feed were not significantly associated with treatment for respiratory disease. A significant
interaction was detected in this model between the shedding of BRCV and seroconversion to the virus, precluding the analysis of the univariate effects of these variables. The acute titer to BRCV was included in the final model to account for cattle which had high titers to BRCV at arrival. Cattle with high acute BRCV antibody titers may have been exposed to the virus but did not seroconvert, even in the face of BRCV infection in the group. In the Texas cattle, no significant effects of acute antibody titers or seroconversion to IBR, BVDV, IBR, or PI3 were found.

Further analysis was performed to account for the combined effects of BRCV shedding and seroconversion. As seen in Table 5.3, cattle who were both shedding the virus and had seroconverted to it after the first month on feed were the most likely of the four classifications to require treatment for respiratory disease, with a risk of 1.6 times that for cattle which did not shed the virus nor seroconvert to it. These cattle also had the lowest average acute BRCV titer. Cattle which were not shedding the virus but had seroconverted to it (OR= 0.59) were the least likely to need treatment. Cattle which neither shed the virus nor mounted a detectable immune response to it had a slightly higher risk (OR=1.0) of developing respiratory disease than those animals which shed the virus but did not produce increased levels of BRCV antibodies (OR= 0.86)

Modeling the effects of BRCV shedding and BRCV seroconversion on average daily gain (Table 5.4), no significant effects were found. Group number and weight at entry were closely associated with average daily gain, with respective p-values of 0.0001 and 0.0066. After accounting for group number, gender was not significant, as many of the groups contained only steers or only heifers. Neither the shedding of BRCV (p=0.67)
nor seroconversion to it (p=0.68) were relevant predictors of average daily gain over the
course of the feeding period.

No other respiratory viruses (IBR, BVDV, BRSV) were found in the subset of
nasal swab supernatants, taken from cattle fed at Lucasville during the fall of 1996, using
virus isolation techniques. However, no testing for bacterial pathogens was performed.
Serum neutralization titers to IBR, PI3, BRSV, and BVDV were available for the Texas
cattle, but had no apparent interaction with BRCV titers, and no significant effect on risk
for treatment for respiratory disease or average daily gain.

5.5 Discussion

Recent investigations have determined that BRCV can be isolated from feedlot
cattle in different geographic locations in the United States,\textsuperscript{12,22,23} and that serological
evidence for BCV infections exists in feedlot cattle in both Canada and the United States.\textsuperscript{22,24} However, the role of BRCV in respiratory disease in feedlot cattle remains unclear.
In our current survey, encompassing 838 cattle in four different feedlots in 2 states, not
only did we find ample evidence of both respiratory tract shedding of BRCV and
seroconversion to it among these cattle, but we were also able to calculate the effects of
BRCV infection on the health and production of these cattle.

In our survey we found 8.1\% of the cattle tested to be shedding BRCV, lower
than the 38\% found by Storz in a 1996 survey. However, no healthy cattle were tested in
this earlier study.\textsuperscript{12} Our finding of seroconversion to BRCV in 58\% of the cattle tested is
similar to Martin et al.'s\textsuperscript{24} 1998 finding of BRCV seroconversion in 61\% to 100\% of
Canadian calves tested. Using multivariable logistic regression to further assess our data
revealed an interaction between the shedding of BRCV and seroconversion to it, when
estimating contributions to the risk of respiratory disease. Thus, the two factors had to be considered together when analyzing their effect on respiratory disease risk.

It is interesting to note that cattle which were both shedding the virus, and had seroconverted to it during the first month on feed, were at increased risk for respiratory disease, as compared to animals which did not shed the virus and/or seroconvert. As the majority of viral shedding took place during the first week on feed, these at-risk animals were most likely infected prior to arrival, and did not mount a protective immune response in time to prevent BRCV infection. This relatively small number of animals introduced the virus to previously-unexposed penmates following the mixing of animals from various sources. By the end of the first month on feed, the majority of cattle had been exposed to the virus and mounted an antibody response to it.

Those cattle which did not shed the virus, but did seroconvert, had the least risk for respiratory disease treatment. There are a number of possibilities as to why no viral shedding was seen in these animals, even though serological evidence of BCV infection was present. Due to the sampling schedules, brief viral shedding may have occurred between sampling dates. It is also possible that these cattle were infected with an enteric strain of BCV (BECV), to which they mounted an antibody response. Previous comparisons of BECV and BRCV strains found them to be antigenically similar, with 6 out of 10 BRCV strains demonstrating similar hemagglutination patterns to those of BECV strains. 27 It is possible that infection with a BECV strain provided immunological cross-protection against infection with a BRCV strain.

Another apparent paradox is seen with the 19 cattle which shed the virus, but which did not seroconvert to it. In addition to the possibilities of either false-positive
antigen ELISA results, or false-negative antibody ELISA results, it is possible that these cattle had relatively high BRCV titers at arrival (they averaged 165) which were protective. It is also possible that these cattle seroconverted sometime after 28 days on feed, when the convalescent samples were taken, or that the cattle were immunosuppressed, not an uncommon finding in feedlot cattle.

No apparent effect of either BRCV shedding or seroconversion to it was seen on average daily gain. Most of the effects of infection would be expected to be seen during the first month on feed, as almost all of the viral shedding was seen during the first week on feed, predominantly during the first 3 days on feed. By the end of the first month on feed, most cattle have been exposed to BRCV and developed antibodies to it, thus preventing a second infection which could significantly reduce the average daily gain after several months on feed. Martin et al. also found that BRCV seroconversion was not related to weight gain, but that higher BRCV arrival titers did result in increased weight gain.24 No such benefit was found in our current study.

In the subset of fall, 1996 Lucasville cattle, no other viral pathogens were found. The treatment rate for respiratory disease in this group was 77%, most likely caused by pneumonias of bacterial origin. As these cattle were vaccinated for BRSV, BVDV, IBR, and PI3, and none of these viruses were isolated from the nasal swabs, it is likely that BRCV worked synergistically with the bacterial agents and physical stresses these cattle were subjected to suppress the immunological defenses of the respiratory tract, allowing bacterial colonization of the lower respiratory tract, leading to pneumonia. With the routine vaccination of feedlot cattle for more common respiratory pathogens, selection may be occurring for previously-unrecognized respiratory viruses, including BRCV.
Although detecting one more factor in the web of causation that leads to bovine respiratory disease complex will not prevent the devastation it causes, developing additional prevention and control measures could help reduce it. BRCV infects feedlot cattle, and its presence appears to increase the risk of developing respiratory disease. Very few cattle arriving at feedlots are effectively vaccinated against BRCV, leaving them susceptible to one more infectious agent at a particularly vulnerable time in their lives.

5.6 Sources and Manufacturers

\(^a\)Minimal Essential Medium, GibcoBRL, Grand Island, NY.

\(^b\)Nunc Maxisorp Microtiter Plate, Nunc, Inc., Naperville, IL.

\(^c\)Nonfat dried milk, Scot Lad Foods, Inc., Pewaukee, WI.

\(^d\)Titertek Plus M96 plate washer, Flow Laboratories Inc., McLean, VA.

\(^e\)Goat anti-guinea pig-IgG-POD, Kirkegaard & Perry Laboratories, Gaithersburg, MD.

\(^f\)Titertek Multiscan plate reader, Flow Laboratories Inc., McLean, VA.

\(^g\)Quattro Pro Windows v. 7.0, Borland International Inc., Scotts Valley, CA.

\(^h\)Immulon IB, Dynex Technologies, Chantilly, VA.

\(^i\)Goat anti-guinea pig POD, Kirkegaard & Perry Laboratories, Gaithersburg, MD.

\(^j\)Emax Precision microplate reader, Molecular Devices, Sunnyvale, CA.

\(^k\)Microsoft Excel 97, Microsoft Corporation, Seattle, WA.
5.7 References


<table>
<thead>
<tr>
<th>Location</th>
<th>Days on Feed Sampled</th>
<th>Number in Herd</th>
<th>BRCV Positive</th>
<th>Percentage of Herd</th>
<th>Percent Seroconverting To BRCV</th>
<th>Number Treated for BRDC</th>
<th>Percent Treated for BRDC</th>
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<tr>
<td>Lucasville-Spr 96 Lot #1</td>
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<td>69</td>
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<td><strong>837</strong></td>
<td><strong>73</strong></td>
<td><strong>8.1</strong></td>
<td><strong>58.0</strong></td>
<td><strong>438</strong></td>
<td><strong>53.7</strong></td>
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*Table 5.1* Sampling schedules, herd size, numbers and percentages of each herd shedding BRCV, BRCV seroconversion rates, and treatment rates for respiratory disease.
<table>
<thead>
<tr>
<th>Variable</th>
<th>Beta</th>
<th>Standard Error</th>
<th>Odds Ratio</th>
<th>p-value</th>
</tr>
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<td>Group*</td>
<td>....</td>
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<tr>
<td>Acute BRCV Titer (per 100 unit change in titer)</td>
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<td>0.01</td>
<td>0.99</td>
<td>0.18</td>
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<tr>
<td>BRCV Shedding</td>
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<td>0.17</td>
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<td>Yes</td>
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<td>No</td>
<td>-0.9993</td>
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<td>Seroconversion to BRCV</td>
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<td>3.13</td>
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Table 5.2 Variables included in the final model for the effects of BRCV shedding and seroconversion on risk of treatment for respiratory disease.
<table>
<thead>
<tr>
<th>BRCV Status</th>
<th>Number of Cattle</th>
<th>Average Acute Titer</th>
<th>Average Convalescent Titer</th>
<th>Beta</th>
<th>Standard Error</th>
<th>OR</th>
<th>95% CI</th>
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</thead>
<tbody>
<tr>
<td>No shedding, no seroconversion</td>
<td>324</td>
<td>879</td>
<td>778</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<td>No shedding, seroconverted</td>
<td>423</td>
<td>92</td>
<td>3497</td>
<td>-0.5201</td>
<td>0.1753</td>
<td>0.59</td>
<td>0.42-0.84</td>
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<td>19</td>
<td>165</td>
<td>245</td>
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<td>49</td>
<td>52</td>
<td>1885</td>
<td>0.4792</td>
<td>0.371</td>
<td>1.6</td>
<td>0.79-3.41</td>
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Table 5.3 The combined effects of BRCV shedding and seroconversion on treatment for respiratory disease.
<table>
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<tr>
<th>Variable</th>
<th>Beta</th>
<th>Standard Error</th>
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Table 5.4 Variables included in the mixed linear model of the effects of BRCV shedding and BRCV seroconversion on average daily gain in feedlot cattle.
CHAPTER 6
EXPERIMENTAL INOCULATION OF CALVES WITH BOVINE RESPIRATORY CORONAVIRUS

6.1 Summary
Enteric isolates of bovine coronavirus (BCV), from both neonatal calf diarrhea and winter dysentery of adult cattle, have previously been inoculated into gnotobiotic and colostrum-deprived calves to determine their pathogenicities and patterns of viremia. With the recent isolation of BCV from the respiratory tracts of feedlot cattle, questions have arisen regarding the possible role of BCV in respiratory disease in older cattle. In order to define the pneumoenteric pathogenicity of BCV strains isolated from feedlot cattle, 13 strains of cell-adapted, cloned bovine respiratory coronaviruses (TC-BRCV) were oronasally inoculated into 14 gnotobiotic and 19 colostrum-deprived calves, which were subsequently monitored for clinical illness and viral shedding from both the enteric and respiratory tracts. Ten of the 14 gnotobiotic (GN) calves developed diarrhea, 11 of them shed virus in their feces, and 5 shed virus in nasal secretions detected by ELISA. In the 19 colostrum-deprived (CD) calves, 9 developed diarrhea, 6 shed virus in their feces, and 2 shed virus from the upper respiratory tract. None of the GN or CD calves developed clinical respiratory disease. Previous inoculation with an enteric BCV (BECV) strain (DB2, isolated from a diarrheic calf) or TC-BRCV isolate prevented viral shedding following challenge with the heterologous BCV strain in 6 GN and 3 CD calves.
Inoculation of cell-adapted, cloned TC-BRCV produced diarrhea and viral shedding patterns similar to those seen with BECV inoculation, but did not produce clinical respiratory disease in the absence of other contributory host or environmental factors. Further challenge studies are needed to determine the contribution of wild-type BRCV or TC-BRCV to respiratory disease when acting synergistically with other infectious agents and physical stresses.

6.2 Introduction

Bovine coronavirus (BCV) was first isolated from calf feces in 1972, and since that time has been shown to cause potentially fatal diarrhea in young calves, primarily 3- to 30-days old. A large, single-stranded, positive-sense enveloped RNA virus, BCV has also been implicated in winter dysentery of adult dairy cattle. The first report of an association of BCV with respiratory disease in calves came in 1982, when Thomas and co-workers conducted a microbiological survey of 8 outbreaks of calfhood pneumonia and found BCV in lung washes and nasopharyngeal swabs taken from calves involved in 2 of the outbreaks. Additional investigations confirmed that BCV could be isolated from young dairy calves showing signs of respiratory disease.

Similar to other coronaviruses, BCV has a tropism for epithelial cells of both the enteric and respiratory tracts. The virus is composed of 4 structural proteins: a 190 kilodalton (kDa) spike (S) protein, a disulfide-linked hemagglutinin-esterase (HE) dimer of 120-140 kDa, a matrix (M) protein of 25 kDa, and a nucleocapsid (N) protein of 50 kDa. The virus causes profuse, watery diarrhea in calves under 30 days of age by attacking epithelial cells throughout the length of the large and small intestines, although histopathologic lesions are most commonly found in the large intestine and cecum.
BCV infection of intestinal cells leads to villous atrophy and fusion, causing malabsorption and increased secretory activity. The resulting dehydration, acidosis, hyperkalemia, hypovolemia, and hypoglycemia can be fatal within 24-48 hours of the onset of diarrhea.  

The consequences of BCV infection of the bovine respiratory tract are less well defined than its effects on the enteric tract. Numerous studies confirmed the ability of the virus to replicate in epithelial cells of the upper respiratory tract, as in a 1984 study in which McNulty et al.\textsuperscript{5} inoculated BCV from a calf with signs of respiratory disease into week-old colostrum-deprived calves. Four of the 7 calves developed either coughs or nasal discharge, and viral shedding in the nasal mucus was found throughout the course of infection in 6 of the 7 calves.\textsuperscript{3} A 1985 study by Reynolds et al.\textsuperscript{6} found respiratory infection of 13 gnotobiotic calves inoculated with both respiratory and enteric field isolates of BCV, but none of these calves developed signs of respiratory disease. Experimental inoculation of 18 gnotobiotic and 7 colostrum-deprived calves by Saif et al.\textsuperscript{10} with a BECV isolate (DB2) resulted in nasal shedding of BCV and profuse diarrhea, but no respiratory disease, although 2 of the calves were found to have focal interstitial emphysema at necropsy. In 1991 Kapil et al.\textsuperscript{11} induced cranioventral interstitial pneumonia, congestion, and hemorrhage in 2 calves via oral inoculation with a pneumoenteric BCV isolated from diarrheic feces.

These earlier studies of BCV in the respiratory tract utilized BCV isolates from outbreaks of either neonatal calf diarrhea or respiratory disease outbreaks in very young calves. Recently, however, investigators have successfully isolated BCV from outbreaks of respiratory disease in older cattle, specifically 6- to 8-month-old cattle entering...
feedlots. It is possible that a respiratory form of BCV (BRCV) could contribute to the economically significant bovine respiratory disease complex (BRDC) of feedlot cattle. BRCV has been associated with BRDC in feedlot cattle, but association is not sufficient to establish causation.

In order to define the possible contribution of BRCV to respiratory disease in cattle, in the absence of potentially protective immune responses and indigenous bacterial flora, strains of BRCV isolated and cloned in cell culture (TC-BRCV) from feedlot cattle were inoculated into gnotobiotic (GN) and colostrum-deprived (CD) calves. Calves were monitored for nasal and fecal shedding of BRCV throughout the course of infection, and enteric and respiratory tissue samples taken at necropsy were examined for the presence of BRCV antigen and histopathologic lesions. Conducting experimental challenges of TC-BRCV in these calves allowed the tissue tropisms, patterns of viremia, and pathogenicity of the viruses to be studied in seronegative animals both with (CD) and without (GN) an indigenous bacterial flora. Additionally, testing of the TC-BRCV isolates in these calves would allow the selection of one or two strains to use in cross-protection studies with enteric BCV (BECV) strains, which have been shown to be antigenically similar to some BRCV strains.

6.3 Materials and Methods

*BRCV Strains:* Nasal swabs were obtained from 838 calves entering feedlots in Ohio and Texas as part of a survey to define the epidemiology of BRCV infections in feedlot cattle. Samples were obtained from all cattle in an incoming group, both clinically ill and apparently healthy animals. The nasal swabs were placed in 4 ml of viral transport medium, then vortexed and centrifuged (1000 x g for 11 minutes) after removing the
swabs. The supernatants were tested for the presence of BRCV using an antigen-capture ELISA. Nasal swab supernatants with corrected absorbances of over 0.1 were inoculated into human rectal tumor (HRT-18) cells, as described previously. Briefly, monolayers of HRT-18 cells were grown in 6-well plates, and washed 3 times with cell culture medium (Eagles minimal essential medium (MEM) containing 1% antibiotics (penicillin, mycostatin, and dihydrostreptomycin) and 1% NaHCO₃). The ELISA-positive nasal swab supernatants were inoculated (0.1 ml) into duplicate wells, and adsorbed for one hour. During this hour, the plates were rotated every 15 minutes, then MEM containing pancreatin (5 µg/ml) was added. The inoculated cultures were incubated for 3 to 4 days in a 5% CO₂ atmosphere, and examined daily for cytopathic effects (CPE).

Liquid limiting dilution was used to clone BRCV strains which successfully infected cell culture. The highest dilution of virus that caused CPE was passaged 3 times in HRT-18 cells.

Experimental Inoculation of Calves: Fourteen gnotobiotic (GN) calves ranging in age from 3- to 41-days and 19 colostrum-deprived (CD) calves ranging in age from 1- to 44-days-old were used in this study. GN calves were obtained via sterile Caesarian-section and housed in sterile isolators. CD calves were bull calves obtained from the Ohio Agricultural Research and Development Center (OARDC) dairy, removed from their dams prior to suckling and housed in isolation rooms at the high security isolation building at OARDC. The BRCV inoculum for each calf consisted of 50 ml of tissue-culture supernatant (TCS), with a minimum tissue culture infectious dose 50/ml (TCID₅₀/ml) of 1x10⁷. Each TC-BRCV strain had been passed only 3 to 6 times in cell
culture. Each calf received 40 ml orally, and 5 ml per nostril. Six calves (B404, B406, CD323, CD324, CD325, CD326) were also given 3 ml of TCS intratracheally (Tables 6.3 and 6.5), and one (CD322) was administered 50 ml of TCS via aerosolization (Table 6.5). Calves were seronegative for BCV prior to initial inoculation. In 9 calves (3 CD and 6 GN, listed in Table 6.6), inoculation with a TC-BRCV or BECV was followed, in 2 to 3 weeks, with challenge inoculation by a heterologous strain. BECV inoculation consisted of 50 ml of either DB2 or DBA fecal suspensions (diluted 1:5), with 40 ml given orally and 5 ml inoculated into each nostril. GN calves B399, B403, B406, B408, and B409, and CD calf 324, were initially inoculated with a TC-BRCV isolate, then challenged with a BECV isolate (either DBA or DB2). Three calves (GN calf B404 and CD calves 317 and 319) were inoculated with the DB2 BECV strain, then challenged with a TC-BRCV isolate. The DB2 strain was also inoculated into 2 naive calves, B405, at 27 days of age, and CD 329, at 1 day of age, to confirm its pathogenicity.

Clinical observation and sample collection: Following inoculation of the calves with BRCV, rectal temperatures (for CD calves only) and clinical signs of respiratory or enteric infection were recorded daily. Nasal swabs and fecal specimens were collected daily, and analyzed for the presence of BRCV using an antigen-capture ELISA. Selected fecal samples were also tested for the presence of BRCV by immunoelectron microscopy (IEM). Fecal samples were assigned a score of 0-4, with 0 being firm; 1, slightly soft; 2, pasty; 3, semiliquid; and 4, liquid. Each calf’s respiratory health was assigned a daily score of 0-3, with 0 being normal; 1, slight nasal discharge; 2, nasal discharge and cough; and 3, dyspnea, rales, and copious nasal discharge. Calves not used
for subsequent challenges were necropsied at 3 to 21 post-inoculation days (PID).

Twenty-one calves were euthanized within 7 days of inoculation, to examine tissues for evidence of acute disease and the presence of BCV antigen. Twelve of the calves were followed for 7 to 43 PID, to study shedding patterns and cross-protection. Duplicate samples of nasal turbinates, trachea, lungs, duodenum, jejunum, ileum, and colon were collected at necropsy for those calves euthanized within 1 to 6 PID. One set of samples was analyzed for the presence of BRCV antigen using a fluorescent antibody assay,\textsuperscript{10} and the other set was submitted to a pathologist for histopathologic examination.

\textbf{BRCV Antigen ELISA:} An indirect, double-antibody sandwich antigen-capture ELISA previously developed by Smith et al.\textsuperscript{14} to detect BCV in fecal samples from adult dairy cattle was adapted to determine the presence of BRCV in nasal swab supernatant fluids. Paired rows of Nunc Maxisorp\textsuperscript{b} 96-well microtiter plates were coated with either 100 µl /well of a 1:8000 dilution of a pool of 3 monoclonal antibodies (one to the BCV S protein, one to the HE protein, and one to the N protein), or a 1:8000 dilution of BCV-antibody negative ascites (SP 2/0), in a carbonate-bicarbonate buffer of pH 9.6. Plates were incubated at 4°C overnight, then rinsed and blocked with 200 µl /well of 5% non-fat dried milk\textsuperscript{c} to reduce non-specific binding. After washing with a Titertek plate washer,\textsuperscript{d} 100 µl of nasal swab supernatant fluids or 100 µl of diluted (1:25) centrifuged (1100xg for 30 minutes) fecal suspensions were applied to 2 BCV antibody-positive coated wells and 2 BCV-antibody negative coated wells. The plates were incubated at room temperature for 1 hour, then rinsed. Guinea pig anti-BCV hyperimmune serum, at 1:2000, was applied to all wells at 100 µl /well for 1 hour at room temperature, followed by
washing. A goat anti-guinea pig horseradish-peroxidase conjugated IgG antibody was applied at a dilution of 1:8000 for 1 hour. To develop color at the end of the assay, 100 μl of a 1:1000 solution of H₂O₂ and 2,2'-azino-bis(3-ethyl-benzthiazoline) sulfonic acid in 0.1 M sodium citrate was applied to each well for 20 minutes. Color development was stopped by adding 50 ml of 5% sodium dodecyl sulfate to each well. The plates were read at 414 nm on a computer-linked ELISA reader, and the optical densities saved as ASCII files. A Quattro Pro spreadsheet program was used to calculate the ELISA values for the samples, by subtracting the average of the paired negative-coated wells from the average absorbance of the paired positive-coated wells. Samples with a resulting absorbance of 0.1 or greater were considered to be positive for BCV.

**Fluorescent antibody assay:** Impression smears of each tissue sampled at necropsy were prepared for fluorescent antibody (FA) assays, as described previously. After air-drying, the tissues were fixed with 100% acetone for 10 minutes at room temperature. The slides were rinsed with PBS and dried, then incubated at 37°C for 1 hour in a humid chamber with fluorescein isothiocyanate (FITC)-conjugated hyperimmune antiserum to the DB2 strain of BCV (diluted 1:10) on one well, and PBS on a control well. After rinsing off the serum, 0.2 ml of PBS was added to each well and incubated for 10 minutes at room temperature. The fixed tissues were examined for immunofluorescence, with the results reported as percentage of cells positive for BCV.

**6.4 Results**

Table 6.1 lists the 13 BRCV strains which were adapted to cell culture from nasal swabs taken from feedlot cattle and subsequently inoculated into GN and CD calves.
Eight other samples, positive for BRCV on ELISA, were passaged in cell culture, but never produced CPE or infected cells FA positive for BCV. The 13 strains which were adapted to cell culture were all isolated from Ohio feedlot cattle. None of the ELISA-positive samples from Texas calves grew in cell culture. Seven of the feedlot calves from which the BRCV strains were isolated were apparently healthy at the time of sampling, and the other 6 were diagnosed as suffering from respiratory disease (defined as coughing, nasal discharge, inappetance, depression, and an elevated rectal temperature.)

As the relative pathogenicities of the various strains were unknown, candidate strains were inoculated into both GN and CD calves. If inoculation of a strain into a naïve calf resulted in no signs of infection (fecal or respiratory shedding, histopathologic lesions, or positive FA results on necropsy), as was the case with TC-BRCV strain #42 (Table 6.2) no further inoculations with that strain were done. Tables 6.2 and 6.3 provide summaries of TC-BRCV inoculation results in 14 GN calves. When a calf was used for subsequent BCV challenge, no FA or histopathology results were available. Eleven of the 14 calves shed the virus in their feces, starting at PID 2-3, and lasting for an average of 3.2 days (range=1-6 days). However, 7 of the 14 GN calves (B371, B373, B392, B394, B398, B401, and B402) were euthanized on or prior to PID 7 to assess acute effects of infection, thus truncating the potential duration of viral shedding and diarrhea. Nine of 14 calves developed diarrhea, usually 1 to 2 days after detection of the virus in the feces by ELISA. The diarrhea persisted from 1 to 6 days, with a mean of 4.1 days.

Five of the 14 GN calves inoculated with TC-BRCV shed the virus in their nasal secretions, starting at PID 2-4 for a duration of 1 to 4 days (mean=2 days), as determined by ELISA, but none of the calves developed signs of respiratory disease. In 2 of these 5
calves, the initial detection of enteric and respiratory shedding occurred simultaneously, in 2 calves the fecal shedding preceded respiratory shedding, and in one calf, detection of respiratory shedding preceded the finding of the virus in the feces by one day.

Fluorescent antibody testing of tissue samples from calves (n=5) necropsied on or before PID 7 (when viral antigen is most frequently present in these tissues) revealed BCV antigen in the enteric tracts of these 5 calves (B 371, B 373, B 392, B 394, and B 398) and 4 of the 5 calves also had BCV antigen in either the nasal turbinates, trachea, or lungs. On necropsy, recently infected calves (less than PID 7) demonstrated mild mucosal petechiation of the distal small intestine and cecum, with distended, thin, fluid-filled intestines. Histopathologic lesions were found in the jejunum and ileum of the calves with BCV-positive FA results, and consisted of villous atrophy and fusion, and crypt abscessation. No gross evidence of respiratory tract infection was found on necropsy. Microscopic lesions of the respiratory tract were found in 3 of the calves ((B371, B401, and B403) and in only one segment of the respiratory tract in each calf.

In the group of CD calves inoculated with BRCV, (Tables 6.4 and 6.5) 6 of 17 calves for whom fecal ELISA results were available shed BRCV in their feces, starting at PID 2-3 and lasting for 3 to 6 days, with an average duration of 3.7 days. Nine of the 19 calves developed diarrhea, beginning on PID 3-4 and persisting for 3 to 5 days (average=2.7 days). Two of the 17 calves for which data were available shed BRCV from their upper respiratory tract, on PID 3-5, and for one day only. None of the challenged CD calves developed clinical signs of respiratory disease.

Eleven of 13 calves with FA results had BRCV antigen in at least one segment of their enteric tract, with the ileum and cecum being the most common locations. BRCV
antigen was present in the respiratory tracts of 7 of 13 calves; in the lower respiratory tract in 2 calves and in the upper respiratory tract (trachea and/or nasal turbinates) in 7 calves. The gross enteric pathology of recently-infected calves was similar to that seen in GN calves, consisting of distended, fluid-filled intestines and sporadic petechiation throughout the distal small intestine and proximal colon. Histopathologic lesions, consisting primarily of villous atrophy, blunting, and fusion, were found in at least one segment of the intestinal tract in 11 of 13 calves for which necropsy samples were available. Three of these calves did not have evidence of BRCV antigen in the enteric tract. No gross respiratory tract lesions were found at necropsy. Nine of the 13 calves had histopathologic lesions in the respiratory tract, most commonly mononuclear or lymphocytic infiltrates of the tracheal lamina propria. One calf, CD307, had atelectic lungs; CD325 was found to have a serofibrinous exudate in its lungs; and CD322 had hemorrhagic lungs, but this finding was determined to be an acute agonal change.

In an attempt to produce respiratory disease, 5 calves (B406, B407, B408, CD327, and CD328) were inoculated with combinations of 3 TC-BRCV strains previously found to differ antigenically from one another. It was hoped that combining the strains would more closely mimic field conditions, where cattle from various sources are mixed at arrival at the feedlot and exposed to different strains of the virus. Fecal shedding of BRCV occurred in 4 of the 5 calves, but respiratory shedding occurred in only 2 of the calves. Four of the 5 calves developed diarrhea, but none showed clinical signs of respiratory disease. Based on each TC-BRCV strain's ability to produce both fecal and nasal shedding of the virus, and evidence of infection in the respiratory and enteric tracts, TC-BRCV strains 117 and 930 were selected for use in future cross-protection studies, in
which BECV-exposed (DB2) calves will be challenged with BRCV, and TC-BRCV-
exposed calves will be challenged with DB2.

In the 9 calves challenged with either BECV or BRCV 2 to 3 weeks after
inoculation with a heterologous strain (Table 6.6), no fecal or respiratory viral shedding
was detected following the challenge exposure, and none of the calves developed
diarrhea after the challenge.

6.5 Discussion

Previous studies have determined that inoculation of CD and GN calves with
enteric isolates of BCV, from either outbreaks of calfhood diarrhea\textsuperscript{10,15,16} or winter
dysentery in adult cattle,\textsuperscript{15} produced both fecal and nasal shedding of the virus. Recently,
investigators have isolated BCV from the upper respiratory tracts of feedlot cattle\textsuperscript{12,13} In
order to determine the potential pathogenicity of these BRCV, thirteen cloned TC-BRCV
isolates from Ohio feedlot cattle were inoculated into GN and CD calves. Fecal and
respiratory shedding of the virus, as well as diarrhea, occurred following primary
inoculation with feedlot BRCV strains, but no respiratory disease was produced.

In both the CD and GN calves, fecal shedding of the virus began on PID 2-3, and
persisted for 3 to 6 days. Diarrhea was first noted 1 to 2 days after the onset of fecal
shedding, and persisted for 3 to 5 days. These findings are similar to those of Saif et al.,\textsuperscript{10}
Heckert et al.,\textsuperscript{17} and El-Kanawati et al.\textsuperscript{15} In a 1986 study, Saif et al.\textsuperscript{10} orally/intranasally
inoculated a group of 6 GN and CD calves with a fecal isolate of BCV (DB2). All of the
challenged calves developed profuse diarrhea by PID 1-4 (mean=PID 2.5) which
persisted for 3 to 7 days, and followed the onset of viral shedding.\textsuperscript{10} Heckert et al.\textsuperscript{17} in
1991 inoculated 5 CD calves with a virulent BECV strain (also DB2), which resulted in
all 5 calves developing profuse, watery diarrhea by PID 2-3, which lasted for 5 to 10
days. These calves also shed BCV in their feces, from 4 to 9 days.\textsuperscript{17} El-Kanawati et al.\textsuperscript{16} found similar ranges for both fecal shedding of BCV (starting at PID 1-3 and lasting for 5
to 6 days) and diarrhea (initiated at PID 2-3 and with a duration of 1 to 3 days) when
cross-challenging calves with a strain of calf diarrhea BCV (DB2) and a winter dysentery
strain (DBA), both of which had been passaged at least 5 times in gnotobiotic calves. It
appears that the fecal shedding patterns, and ability to produce diarrhea in GN and CD
calves, are very similar for BECV and TC-BRCV strains.

Similar patterns of BCV shedding from the upper respiratory tract were also seen
in this study, as compared to previous studies of BECV isolates. As in the 1986 study by
Saif et al.,\textsuperscript{16} BCV-infected nasal epithelial cells were most commonly found on PID 2 to
4, using direct FA staining rather than ELISA, and no clinical signs of respiratory disease
developed. Heckert et al.\textsuperscript{17} found more prolonged shedding of BCV from the respiratory
tract (also using direct FA assays, rather than ELISAs), with a range of 6 to 11 days, and
an average of 8.4 days. As in the other studies, no signs of respiratory disease were
detected.\textsuperscript{17} In El-Kanawati et al.'s 1996 study,\textsuperscript{16} nasal shedding was first detected on PID
1 to 4, using both FA assays and ELISAs, as in the current study, but the shedding
persisted far longer, from 8 to 12 days, rather than the 1 to 4 days found in this study. It is
possible that the shorter duration of nasal shedding following TC-BRCV inoculation may
be due to strain differences between BECV and BRCV isolates, or it may be a function of
different sensitivities of the assays used (FA vs. ELISA). Alternatively, the adaptation,
cloning, and passage of the TC-BRCV in cell culture, although only low level cell
passages were used (<6), may have altered the virulence of the wild type BRCV strains,
or selected for less virulent strains by cloning in cell culture. Whether several sequential passages of the TC-BRCV strains in calves would restore the respiratory virulence is unknown. The use of RT-PCR to detect BCV in nasal secretions may offer improved sensitivity in monitoring both respiratory and enteric shedding of the virus.\(^{18}\)

In 1995 Stine et al.\(^{19}\) reported 3 of 4 gnotobiotic calves inoculated with a BRCV isolate showed signs of mild interstitial pneumonia at necropsy (at PID 4 to 10), as well as demonstrating histologic evidence of bronchiolitis and bronchiolar necrosis. One of these calves’ lungs and nasal turbinates were also positive for BCV, using immunohistochemical staining, on PID 4, similar to the current findings of BCV antigen in lungs, turbinates, and tracheas of calves necropsied within 3 to 7 days of BCV inoculation. BCV replicates in the respiratory tracts of gnotobiotic and colostrum-deprived calves, but the pathologic effects of that replication vary.

It appears that inoculation of a GN or CD calf with a TC-BRCV isolate prevents viral shedding and diarrhea when the calf is challenged 2 to 3 weeks later with a BECV strain, and that BECV exposure protects against viral shedding and diarrhea following challenge with a TC-BRCV strain (Table 6.6). Reynolds found that GN calves infected with BECV strains were resistant to challenge with certain wild-type BRCV strains, and that antisera to 9 different BECV and BRCV strains effectively neutralized the virus in homologous sera, and cross-reacted with the 8 other isolates.\(^6\) Hasoksuz et al.\(^{13}\) found that 6 of the TC-BRCV strains used in this study were similar to 2 calf diarrhea and 2 winter dysentery BECV strains, based on hemagglutination patterns and receptor-destroying enzyme activity. Nine of 10 tested TC-BRCV isolates were similar to BECV subtypes, based on virus neutralization and hemagglutination-inhibition patterns.\(^{13}\) It is
very likely that infection with a BECV strain will protect against infection with a TC-BRCV strain, and vice versa. A study is currently in progress to test this hypothesis in CD calves. Four calves will be initially exposed to DB2 and monitored for illness and viral shedding. Two weeks after the primary inoculation, the calves will be challenged with BRCV 930, and once again followed for evidence of infection. Another 4 calves will initially be inoculated with BRCV 930, and challenged after 2 weeks with DB2, in order to study the effects of cross-protection in a controlled study.

Although infection with both BECV and TC-BRCV isolates resulted in detection of the virus in nasal secretions, neither type of isolate produced clinical respiratory disease. In this current study, inoculation with 9 of the 13 TC-BRCV strains resulted in BRCV antigen being detected in the respiratory tract at necropsy, and 7 of the 13 strains resulted in mild histopathologic lesions of the respiratory tract. However, no clinical signs of respiratory disease, including coughing, nasal discharge, and dyspnea, were detected at any time during the course of infection. The gross pathology seen in the enteric tracts was similar to that resulting from BECV infection of young calves\(^1\), and would not be pathognomonic for BRCV infection.

Viewed in the context of the complex causes of respiratory disease and their interactions with one another, it is not surprising that this single agent, acting alone, was not able to reproduce respiratory disease. The bovine respiratory disease complex (BRDC) of feedlot cattle arises from the interaction of viruses, physical stresses, and bacteria, and cannot be attributed to a single causative agent. The generally accepted web of causation for BRDC consists of a combination of viruses, including bovine herpesvirus-1 (BHV-1), bovine respiratory syncytial virus (BRSV), parainfluenza-3.
(PI3), and bovine virus diarrhea virus (BVDV) and multiple environmental stresses, including transportation, mixing of cattle from different sources, rapid changes in ration, vaccination, and inclement weather, all working together on susceptible cattle to overwhelm the innate immunological defenses of the respiratory tract.\textsuperscript{20,21,22} With the normal immune response suppressed, normally commensal organisms of the upper respiratory tract, such as \textit{Pasteurella hemolytica} and \textit{P. multocida}, can colonize the lower respiratory tract and cause potentially fatal fibrinous bronchopneumonia.\textsuperscript{20}

One of these component causes, in the absence of the others, may not be adequate to produce significant respiratory disease. Numerous investigators have found that experimental inoculation of a single virus, known to contribute to severe cases of respiratory disease in field settings, did not result in clinical signs of equal severity.\textsuperscript{23,24,25} Straub\textsuperscript{23} was not able to reproduce respiratory disease in healthy seronegative cattle via intranasal inoculation of BRSV. Using a BRSV isolate from a calf suffering from acute respiratory disease, an isolate which was not passaged in cell culture, van der Poel et al.\textsuperscript{24} were able to cause increased respiratory rates and elevated rectal temperatures in 2 groups of 6 gnotobiotic calves, but reported that the clinical signs were not as severe as those seen in field cases. In 1998, Broderson et al.\textsuperscript{25} reported more severe clinical signs of respiratory disease in 5 calves concurrently infected with BRSV and BVDV than in 2 groups of 5 calves each, which were inoculated with only a single virus. At necropsy, more severe lesions of the respiratory and enteric tracts were found in the dual-infected calves.

IBR infections, in the absence of additional viral or secondary bacterial infections, result in normal lungs, causing clinical signs referable to the upper respiratory tract only.
The PI-3 virus, while commonly isolated from newly arrived feedlot cattle, causes only mild to inapparent clinical signs of respiratory disease. The possible mechanisms of potentiation between infectious agents are numerous. It appears that IBR virus increases the activity of the enzyme elastase in nasal mucus, which exposes the cell surface receptors of nasal epithelial cells needed for colonization of the cells by *P. hemolytica*. Viral infections can increase the levels of divalent cations, including iron and zinc, in the respiratory tract, which improve the adherence of bacteria. Viruses can also interfere with lymphocyte and alveolar macrophage function, further decreasing the clearance of bacteria from the respiratory tract.

It is thus possible that BRCV does contribute to the development of respiratory disease, by acting synergistically with the many other "causes" of respiratory disease. Tsunemitsu et al. enhanced the ability of BCV to produce diarrhea in adult cattle by administering ice water and dexamethasone to the cattle, re-creating host and environmental factors which contribute to the natural or field development of winter dysentery in adult dairy cattle. Similar challenge studies are needed to determine the effects of BRCV on the bovine respiratory tract when acting synergistically with other infectious agents and physical stresses.

### 6.6 Sources and Manufacturers

*a* Minimum essential medium, Gibco BRL, Grand Island, NY.

*b* Nunc Maxisorp Microtiter Plate, Nunc, Inc., Naperville, IL.

*c* Nonfat dried milk, Scot Lad Foods, Inc., Pewaukee, WI.

*d* TiterTek Plus M96 plate washer, Flow Laboratories Inc., McLean, VA.
6.7 References


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<td>Respiratory Disease</td>
</tr>
<tr>
<td>220</td>
<td>Lucasville 1996</td>
<td>Healthy</td>
</tr>
<tr>
<td>228</td>
<td>Lucasville 1996</td>
<td>Healthy</td>
</tr>
<tr>
<td>255</td>
<td>Lucasville 1996</td>
<td>Respiratory Disease</td>
</tr>
<tr>
<td>265</td>
<td>Lima 1995</td>
<td>Healthy</td>
</tr>
<tr>
<td>345</td>
<td>Wooster 1995</td>
<td>Respiratory Disease</td>
</tr>
<tr>
<td>430</td>
<td>Lucasville 1995</td>
<td>Respiratory Disease</td>
</tr>
<tr>
<td>440</td>
<td>Lucasville 1995</td>
<td>Respiratory Disease</td>
</tr>
<tr>
<td>930</td>
<td>Lucasville 1996</td>
<td>Respiratory Disease</td>
</tr>
</tbody>
</table>

Table 6.1 BRCV strains grown in cell culture which were inoculated into GN and CD calves. The year and Ohio feedlot location where each sample originated are included, as is the respiratory disease status of the calf from which each sample was taken, at the time the sample was obtained.
<table>
<thead>
<tr>
<th>GN Calf ID</th>
<th>BRCV Strain</th>
<th>Age in days at inoculation</th>
<th>ELISA Fecal Shedding PID (duration)</th>
<th>IEM Fecal Shedding PID</th>
<th>ELISA Nasal Shedding PID (duration)</th>
<th>PID Diarrhea PID (duration)</th>
<th>PID Respiratory Disease</th>
<th>PID at necropsy</th>
<th>FA Positive</th>
<th>Histopatological Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>B 371</td>
<td></td>
<td>213</td>
<td>41</td>
<td>2</td>
<td>LIC</td>
<td>No</td>
<td>3</td>
<td>No</td>
<td>3</td>
<td>D,J,I,C, Turb.</td>
</tr>
<tr>
<td>B 373</td>
<td></td>
<td>265</td>
<td>21</td>
<td>2</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>3</td>
<td>D,J,I,C, Lung</td>
</tr>
<tr>
<td>B 392</td>
<td></td>
<td>228</td>
<td>27</td>
<td>2</td>
<td>No</td>
<td>4</td>
<td>1</td>
<td>No</td>
<td>5</td>
<td>D,J,I,C, Turb, Trach.</td>
</tr>
<tr>
<td>B 394</td>
<td></td>
<td>117</td>
<td>30</td>
<td>2</td>
<td>LIC</td>
<td>No</td>
<td>2</td>
<td>No</td>
<td>4</td>
<td>D,J,I,C, L,T,T, J,I</td>
</tr>
<tr>
<td>B 398</td>
<td></td>
<td>220</td>
<td>29</td>
<td>2</td>
<td>LIC</td>
<td>No</td>
<td>4</td>
<td>No</td>
<td>4</td>
<td>D,J,I,C, No, Ileum</td>
</tr>
<tr>
<td>B 399</td>
<td></td>
<td>67</td>
<td>26</td>
<td>2</td>
<td>No</td>
<td>3</td>
<td>3</td>
<td>No</td>
<td>17</td>
<td>NA, NA, No</td>
</tr>
<tr>
<td>B 401</td>
<td></td>
<td>76</td>
<td>24</td>
<td>2</td>
<td>No</td>
<td>2</td>
<td>3</td>
<td>No</td>
<td>7</td>
<td>NA, NA, Turb</td>
</tr>
</tbody>
</table>

**Table 6.2** Fecal and respiratory viral shedding, clinical illness, and necropsy results for 7 of 14 gnotobiotic calves inoculated with BRCV field isolates. LIC=large intestine contents, D=duodenum, J=jejenum, I-ileum, C=colon, L=lung, T=turbinate, Tr=trachea, PID=post-inoculation day, NA=not applicable (FA is not applicable for samples collected greater than 7 PID).
<table>
<thead>
<tr>
<th>GN Calf ID</th>
<th>BRCV Strain</th>
<th>Age in days at inoculation</th>
<th>ELISA Fecal Shedding PID (duration)</th>
<th>IEM Fecal Shedding PID</th>
<th>ELISA Nasal Shedding PID</th>
<th>Diarrhea PID</th>
<th>Respiratory Disease PID</th>
<th>PID at necropsy</th>
<th>FA Positive</th>
<th>Histo Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>B 402</td>
<td>42</td>
<td>27</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>5</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>B 403</td>
<td>930</td>
<td>8</td>
<td>3 (3)</td>
<td>3</td>
<td>8 (1)</td>
<td>No</td>
<td>No</td>
<td>28</td>
<td>NA</td>
<td>NA Colon Lung</td>
</tr>
<tr>
<td>B 404</td>
<td>220</td>
<td>5</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>43</td>
<td>NA</td>
<td>NA NA NA NA</td>
</tr>
<tr>
<td>B 406</td>
<td>76,117, 220</td>
<td>4</td>
<td>2 (5)</td>
<td>2</td>
<td>2 (3)</td>
<td>4 (6)</td>
<td>No</td>
<td>40</td>
<td>NA</td>
<td>NA No No</td>
</tr>
<tr>
<td>B 407</td>
<td>430,440, 228</td>
<td>23</td>
<td>4 (1)</td>
<td>4</td>
<td>No</td>
<td>4 (6)</td>
<td>No</td>
<td>17</td>
<td>NA</td>
<td>NA NA</td>
</tr>
<tr>
<td>B 408</td>
<td>430,440, 228</td>
<td>27</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>3 (4)</td>
<td>No</td>
<td>28</td>
<td>NA</td>
<td>NA NA</td>
</tr>
<tr>
<td>B 409</td>
<td>220</td>
<td>3</td>
<td>3 (6)</td>
<td>6</td>
<td>2 (1)</td>
<td>4 (5)</td>
<td>No</td>
<td>34</td>
<td>NA</td>
<td>NA NA</td>
</tr>
</tbody>
</table>

Table 6.3 Fecal and respiratory viral shedding, clinical illness, and necropsy results for 7 of 14 gnotobiotic calves inoculated with BRCV field isolates. LIC=large intestine contents, D=duodenum, J=jejenum, I-ileum, C=colon, L=lung, T=turbinate, Tr=trachea, PID=post-inoculation day, NA=not applicable (FA is not applicable for samples collected greater than 7 PID, and histology not available for calves used for subsequent challenges).
Table 6.4: Fecal and respiratory viral shedding, clinical illness, and necropsy results for 10 of 19 colostrum-deprived calves inoculated with BRCV field isolates. LIC=large intestine contents, D=duodenum, J=jejenum, I=ileum, C=colon, L=lung, T=turbinate, Tr=trachea, PID=post-inoculation day, NA=not applicable (FA is not applicable for samples collected greater than 7 PID, and histology and FA not available for calves used for subsequent challenges).
<table>
<thead>
<tr>
<th>Calf ID</th>
<th>BRCV Strain</th>
<th>Age in days at inoculation</th>
<th>Fecal Shedding PID (duration)</th>
<th>Fecal Shedding PID</th>
<th>Nasal Shedding PID (duration)</th>
<th>PID Diarrhea (duration)</th>
<th>PID Respiratory Disease</th>
<th>PID at necropsy</th>
<th>FA Positive Enteric</th>
<th>Histo Lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD 320</td>
<td>255</td>
<td>23</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>9</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CD 321</td>
<td>430</td>
<td>23</td>
<td>2 (3)</td>
<td>2</td>
<td>No</td>
<td>2 (3)</td>
<td>No</td>
<td>4</td>
<td>D,J,I,C</td>
<td>Trileum, Trach</td>
</tr>
<tr>
<td>CD 322</td>
<td>117</td>
<td>27</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>4</td>
<td>J,I,C</td>
<td>L, Tr, T, Trach</td>
</tr>
<tr>
<td>CD 323</td>
<td>255</td>
<td>24</td>
<td>1 (3)</td>
<td>NA</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>3</td>
<td>Ileum</td>
<td>No</td>
</tr>
<tr>
<td>CD 324</td>
<td>117</td>
<td>24</td>
<td>1 (5)</td>
<td>2</td>
<td>1 (1)</td>
<td>3 (4)</td>
<td>No</td>
<td>20</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CD 325</td>
<td>220</td>
<td>17</td>
<td>2 (2)</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>3</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>CD 326</td>
<td>67</td>
<td>24</td>
<td>1 (1)</td>
<td>No</td>
<td>No</td>
<td>4 (4)</td>
<td>No</td>
<td>7</td>
<td>I,C</td>
<td>Turbinate, Ileum, Trach</td>
</tr>
<tr>
<td>CD 327</td>
<td>76,117, 220</td>
<td>24</td>
<td>No</td>
<td>3, Ileum</td>
<td>No</td>
<td>4 (1)</td>
<td>No</td>
<td>4</td>
<td>D,J,I,C</td>
<td>Trach, I, C</td>
</tr>
<tr>
<td>CD 328</td>
<td>76,117, 220</td>
<td>24</td>
<td>4 (1)</td>
<td>3</td>
<td>No</td>
<td>4 (1)</td>
<td>No</td>
<td>4</td>
<td>D,J,I,C</td>
<td>Trach, I, No</td>
</tr>
</tbody>
</table>

Table 6.5 Fecal and respiratory viral shedding, clinical illness, and necropsy results for 9 of 19 colostrum-deprived calves inoculated with BRCV field isolates. LIC=large intestine contents, D=duodenum, J=jejunum, I=ileum, C=colon, L=lung, T=turbinate, Tr=trachea, PID=post-inoculation day, NA= not applicable (FA is not applicable for samples collected greater than 7 PID, and histology and FA not available for calves used for subsequent challenges).
<table>
<thead>
<tr>
<th>Calf ID</th>
<th>BCV Strain</th>
<th>Inoculation</th>
<th>Challenge Exposure</th>
<th>Days to Challenge</th>
<th>Diarrhea</th>
<th></th>
<th>Fecal Shedding</th>
<th>Nasal Shedding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Onset PID</td>
<td>Duration (days)</td>
<td>Onset PID</td>
<td>Duration (days)</td>
</tr>
<tr>
<td>B 399</td>
<td>BRCV 67</td>
<td>DBA</td>
<td>14</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>No</td>
</tr>
<tr>
<td>B 403</td>
<td>BRCV 930</td>
<td></td>
<td></td>
<td></td>
<td>No</td>
<td>No</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>B 406</td>
<td>BRCV 76,</td>
<td>DB2</td>
<td>14</td>
<td>4</td>
<td>6</td>
<td>2</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>117,220</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>B 408</td>
<td>BRCV 430,</td>
<td>DB2</td>
<td>14</td>
<td>3</td>
<td>4</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>440,228</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>B 409</td>
<td>BRCV 220</td>
<td>DB2</td>
<td></td>
<td>4</td>
<td>5</td>
<td>3</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>CD 324</td>
<td>BRCV 117</td>
<td>DB2</td>
<td>14</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>B 404</td>
<td>DB2</td>
<td>BRCV 220</td>
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<td>No</td>
<td>3</td>
<td>3</td>
<td>No</td>
</tr>
<tr>
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<td>DB2</td>
<td>BRCV 76</td>
<td>21</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>CD 319</td>
<td>DB2</td>
<td>BRCV 67</td>
<td>14</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>B 405</td>
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<td>none</td>
<td>14</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 6.6 Development of diarrhea, fecal and nasal shedding of BCV by gnotobiotic or colostrum-deprived calves inoculated or challenged with enteric or respiratory BCV strains. Fecal shedding was detected using ELISA or IEM, nasal shedding was detected using ELISA. PID=post-inoculation day.
As Louis Pasteur's germ theory of disease causation became increasingly popular in the scientific community, it appeared that finding the causes of all manner of disease would now be quite simple. Simply isolate the agent from that disease, and you have your cause. Jakob Henle and Robert Koch established postulates of infectious disease causation based on germ theory which are still referred to today. To attribute the cause of a disease to a microorganism, that microorganism must occur in every case of the disease of interest, occur in no other disease as a nonpathogenic parasite, and, following isolation from a case of the disease and growth in pure culture, it can produce the disease of interest in a susceptible patient. These postulates were invaluable in establishing causation in a large number of diseases, but also suffered from limitations, as in the case of disease complexes with multiple causes.

Bradford Hill expanded the concept of disease causation by offering 9 criteria of causation, not all of which were necessary or sufficient for establishment of causation, but which did provide useful guidelines in the investigation of causal hypotheses. These criteria included: the strength of the observed association, the consistency of the association, specificity of an effect, temporal precedence, existence of a biological
gradient, plausibility of the association, coherence of the hypothesis with those of other investigators, experimental evidence of causation, and the analogy of similar causal pathways. 2

The investigation of a purported component cause of a disease with many possible causes draws heavily from Hill's list of criteria. The strength and nature of an association between a possible cause and the disease of interest needs to be established, using both observational and experimental evidence, in order to determine if further investigation is warranted. Bovine respiratory disease complex (BRDC) is an example of a disease with multiple causes, most of which, as defined by Rothman, are components of sufficient cause, but are not, in and of themselves, sufficient causes. 3 A sufficient cause is one which inevitably produces the observed effect, but it is rare that a single agent would act alone as a sufficient cause. 3 Understanding the "cause" of BRDC consists of identifying component causes which, when acting together, produce the observed morbidity and mortality.

Many component causes of BRDC have been identified, including viruses (infectious bovine rhinotracheitis, bovine respiratory syncytial virus, bovine virus diarrhea virus, and parainfluenza virus), bacteria (Pasteurella hemolytica and P. multocida), and stress (transportation, mixing of cattle, changes in ration, vaccination). 4,5 Even with improvements in prevention and control measures aimed at these component causes, BRDC continues to occur, causing significant financial losses to the feedlot industry. Although identifying yet another component cause would be unlikely to prevent the occurrence of BRDC, it is possible that taking steps to minimize its impact may reduce the incidence or severity of cases that occur.
Bovine coronavirus (BCV) has been implicated as a possible component cause of BRDC, as it had been isolated from feedlot cattle suffering from respiratory disease and is known to have a tropism for the bovine respiratory tract. In order to better define the nature of the association between BCV and BRDC, we collected the observational and experimental data presented here. In order to survey large numbers of feedlot cattle for the presence of bovine respiratory coronavirus (BRCV) and antibodies to it, we adapted two enzyme-linked immunosorbent assays (ELISAs), which had previously been developed for the detection of bovine enteric coronavirus (BECV) in adult dairy cattle. Using a pool of 3 monoclonal antibodies to 3 different proteins of BCV, the antigen-capture ELISA was adapted to detect BRCV in nasal swabs from cattle. The same pool of monoclonals was used in an indirect antibody-detection ELISA, adapted to determine the levels of anti-BRCV antibodies in serum samples from feedlot cattle.

With appropriate diagnostic assays in place, we surveyed 1074 feedlot cattle, entering 7 different feedlots in 12 separate groups, for the presence of BRCV antigen and antibodies, as well as collecting information on treatment for respiratory disease and production, as evaluated by average daily gain (ADG). All cattle in incoming groups were sampled, both those that appeared healthy and those that were suffering from respiratory disease. The percentage of cattle in each group shedding the virus ranged from 0 to 35.9%, with an overall rate of 7.3%. Most of the viral shedding (94%) occurred during the first week on feed. Seroconversion to BRCV (defined as a four-fold or greater increase in BRCV ELISA antibody titer) occurred in 58% of the cattle surveyed, with ranges for individual groups from 20.3% to 84.1%.
Health and production data were available on a subset of 838 cattle. Using multivariable linear regression to assess the effects of BRCV shedding and seroconversion on treatment for respiratory disease, it was found that a significant interaction existed between BRCV shedding and seroconversion. Cattle shedding the virus and developing a seroresponse to it were 1.6 times more likely to need treatment for respiratory disease than cattle which neither shed the virus nor seroconverted to it. Neither BRCV shedding nor seroconversion to it exerted any apparent effect on ADG, as determined by multivariable linear regression.

Thirteen BRCV strains were isolated from the feedlot cattle, and were cultured in HRT-18 cells. A colleague antigenically characterized these isolates and compared them to previously isolated (BECV) strains, finding most of the BRCV strains to be similar to the BECV strains. Inoculating the BRCV isolates into gnotobiotic (GN) and colostrum-deprived (CD) calves resulted in diarrhea and viral shedding patterns similar to those produced by BECV inoculation. Although the inoculated calves developed diarrhea, and shed BRCV from the enteric and respiratory tracts, no signs of respiratory disease were observed. Other host and environmental factors are needed to reproduce clinical respiratory disease in these calves, as BRCV does not appear able to reproduce clinical respiratory disease as a sole pathogen.

The available evidence points to BRCV as a component, but not sufficient, cause of BRDC. There is an association between BRCV infection and BRDC, the proposed association is plausible, given the contributions of other viruses to BRDC, the findings of our investigation are coherent with previous findings (Storz et al.), and similar causal pathways have been found. BRCV acts synergistically with other viruses, bacteria, and
environmental stresses to contribute to the development of respiratory disease.

Vaccinating calves for BRCV would not eliminate BRDC, but it may help reduce the incidence and severity by removing one more component cause.

References


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