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WINTER DYSENTERY IN DAIRY CATTLE: AN EPIDEMIOLOGICAL INVESTIGATION OF THE ROLE OF BOVINE CORONAVIRUS AND OTHER RISK FACTORS

DISSERTATION

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

By

David Roy Smith, B.S., D.V.M.

* * * * *

The Ohio State University
1997

Dissertation Committee:
Professor Linda J. Saif, Advisor
Professor Kent H. Hoblet, Co-advisor
Professor Donald R. Redman
Assistant Professor Paul S. Morley
Assistant Professor Thomas E. Wittum

Approved by

Linda J. Saif
Advisor

Kent H. Hoblet
Co-advisor
Veterinary Preventive Medicine
Graduate Program
ABSTRACT

Winter dysentery is characterized by the acute onset of diarrhea, rapidly spreading through adult cattle herds. The causes of winter dysentery are unknown, but bovine coronavirus infection has been demonstrated in affected herds. Other suggested risk factors relate to host immunity and environmental conditions. The objectives of this research were: 1) to develop and evaluate bovine coronavirus diagnostic assays for investigating winter dysentery; 2) test the causal role of bovine coronavirus in winter dysentery; 3) and to generate new, or strengthen older, causal hypotheses about winter dysentery.

A bovine coronavirus antigen capture ELISA was 96% sensitive and 100% specific when testing the feces from diarrheic neonatal calves. However, the assay demonstrated moderate agreement (kappa = 0.50) with immunoelectron microscopy testing of feces from adult cattle in winter dysentery affected herds. Several ELISA positive, and 2 ELISA negative, but immunoelectron microscopic positive fecal samples were demonstrated to contain infectious bovine coronavirus particles following inoculation into colostrum-deprived calves. Antibody-complexed virus particles may explain the false negative ELISA results.

Bovine coronavirus IgG antibody ELISA seroresponses demonstrated moderate
agreement with immunoelectron microscopic examination of the feces, but the agreement varied with the individuals acute BCV antibody titer. Better agreement was obtained at acute titer values >1100, than below.

Twelve winter dysentery-affected, and 24 unaffected herds were studied for herd-level risk factors in a case-control field investigation. Higher herd prevalence of bovine coronavirus seroresponses was associated with greater risk for winter dysentery, as was higher bovine viral diarrhea virus seroresponse prevalence, tied housing of cattle rather than freestall housing, and the use of manure handling equipment to handle feed.

Within the affected herds, 125 sick individuals and 104 non-sick controls were studied for cow-level risk factors. Pregnant individuals had 0.49 odds of being sick compared to non-pregnant herdmates. The odds of being sick was greater in individuals with higher antigen-capture ELISA values. And the odds of being sick among individuals seroresponding to BCV was greater with higher acute BCV antibody titers, while the odds of being sick among individuals not seroresponding to BCV was greater with lower acute BCV antibody titers.
Dedicated to Fay, Emily, and Sarah
ACKNOWLEDGMENTS

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VITA

July 5, 1958 ............................................... Born - Dayton, Ohio

1980 .......................................................... B.S., Agriculture, The Ohio State University

1983 .......................................................... D.V.M, The Ohio State University

1983 - 1992 ............................................... Private veterinary practice, Spring Meadow Veterinary Clinic, Inc. Ashland, Ohio

1992 - 1993 ................................................ Graduate Research Associate, OARDC, The Ohio State University

1993 - 1994 ................................................ Clinical Instructor House Staff, OARDC, The Ohio State University

1994 - present ............................................ Graduate Research Associate, OARDC, The Ohio State University

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FIELDS OF STUDY

Major Field: Veterinary Preventive Medicine

Studies in epidemiology, biostatistics, immunology, infectious diseases.
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INTRODUCTION

Winter dysentery is a disease of dairy and beef cattle, characterized by an acute herd outbreak of diarrhea affecting primarily the adult herd members. Few animals die, but the disease is costly to farmers because of the severe decreases in milk production and lost body condition in affected individuals. Scientific investigation of the cause of winter dysentery began over 65 years ago and continues to this day. Currently the causal factors of winter dysentery are poorly understood. Chapter 1 of this dissertation reviews previous investigations of winter dysentery. The unfolding story of winter dysentery is intriguing for several reasons. First, winter dysentery is one of the last ‘classic’ infectious diseases of cattle for which the etiology remains elusive. Previous investigators have demonstrated considerable prowess of observation, yet the story illustrates how easily one may be mislead when attempting to demonstrate causal associations. Also the story serves to remind us to be cautious in our inferences because an incorrect inference can remain in the literature for many years. Finally, I believe that the story also illustrates that the keys to understanding the mechanisms of health and disease are not within the domain of a single discipline, and it is through the integration of the work of many, from a variety of disciplines and backgrounds, that these secrets are unlocked.
Researchers from many parts of the world have provided evidence that bovine coronavirus may be associated with winter dysentery, yet the nature of this association is not clear. Chapter 2 reviews some of what is known about bovine coronavirus, its associated diseases and pathogenesis. Some epidemiological features of bovine coronavirus associated diseases lead to speculation that persistent viral infection may in part explain our observations. It is clear that understanding the mechanisms of coronavirus infection is critical to effectively testing causal hypotheses about this agent and winter dysentery. Bovine coronavirus is a difficult agent with which to work; the agent is difficult to cultivate, and its morphology can be confused with cellular organelles and fecal debris. The development of reliable diagnostics for bovine coronavirus exposure is therefore necessary before a meaningful causal investigation of its role in winter dysentery can be undertaken.

It has not proven simple to understand or demonstrate the causal pathways involved in winter dysentery, although many clues have been discovered. Chapter 3 reviews some of the concepts of causation, and how cause is demonstrated, as it concerns philosophers, epidemiologists, and microbiologists. Understanding the strengths and limitations of causal criteria is a prerequisite to causal inference in etiologic investigations.

The objectives of this scientific inquiry were threefold:
1) To develop and evaluate diagnostic assays to measure bovine coronavirus exposure for use in causal investigations of winter dysentery. To be useful these assays should:
a) be specific for bovine coronavirus, but sensitive enough to identify infection by all known strains of the virus; b) be applicable to large numbers of samples required for epidemiological investigations.

2) To specifically test the hypothesis that bovine coronavirus infection is causally associated with winter dysentery outbreaks. To adequately test this hypothesis, it is necessary to evaluate separately the risk factors for the herd, and for the individual.

3) To generate new, or strengthen older, causal hypotheses about winter dysentery. Associations noted by previous researchers and empirical hypotheses based on practice observations will be tested at the appropriate level of investigation.

The remaining chapters are devoted to detailing these investigations, and to summarizing the results and conclusions.
CHAPTER 1

THE SEARCH FOR THE CAUSE OF WINTER DYSENTERY

There have been many synonyms for the disease, now termed winter dysentery (WD) including enzootic diarrhea, infectious diarrhea, winter scours, black scours, barn scours, bovine virus enteritis, infectious diarrhoea of dairy cattle, bovine enzootic enteritis, and epizootic diarrhea. The term 'winter dysentery' was in use by 1931 but did not appear frequently in the veterinary literature until years later and now appears to be the commonly used and accepted term for the syndrome. This chapter addresses the 65 year history of WD investigation and the search for its cause.

1.1 Descriptive epidemiology

Early in this century, bloody dysentery of dairy and beef cattle was recognized, though poorly characterized in regards to the epidemiology of the disease within or among herds. In 1917, Steffen described an acute diarrheal disease of cattle herds, typically lasting 3-10 days; affected individuals had semi-fluid, deep chocolate diarrhea and usually developed a dry cough. This disease, of unknown etiology affected cattle of all ages, was rarely fatal, most often occurred during the winter months, and was confined to the North Central United States. In 1931, Jones and Little reported an
investigation of 5 herds experiencing outbreaks of diarrhea of adult dairy cattle to which they attributed a vibrio organism as the cause. The disease had been previously recognized in the area and was often called winter dysentery. In 1933 they summarized 3 years of epidemiological investigation of the disease. They described a contagious disease occurring from November to March with characteristic clinical signs:

The animals become dull, refuse food and may exhibit signs of abdominal pain. There is little or no elevation in temperature although in a few cases low fever has been noted. The onset may be accompanied by a dry cough and excessive salivation. The milk yield drops markedly. The outstanding symptom is diarrhea; the feces are liquid and usually dark brown or greenish-black in color. Large quantities of blood and blood stained mucus may be passed. In certain cases mucous casts a meter or more in length have been found in the stools. The disease may run a relatively short course (a few hours) or diarrhea may persist for 4 or 5 days. The rapidity of recovery and the loss in general condition will depend on the length and severity of the diarrhea. In general there is little mortality except in isolated instances and these usually in small herds where little interchange cows has previously taken place. Among highly susceptible individuals the disease is extremely severe and apt to result in mortality.

In 1948, Rollinson reported similar epidemics of adult cow diarrhea in England affecting at least 760 animals in 41 herds. His survey of practicing veterinarians suggested that the condition may have existed there for as much as 20 years. Practitioners were in agreement that this was a disease of adult cattle, rarely affecting calves; and most commonly seen from November to March. After closely following 4 herds prospectively, Rollinson felt there were 6 pathognomonic features of the disease he described:

1. Sudden onset of watery, foetid, bubbly diarrhoea.
2. Swift spread amongst adult animals in contact.
3. By the time of investigation the temperature of an affected animal is often normal.
5. Spontaneous recovery with little change in the general condition of the animal
6. No explanation of the condition by examination of feeding-stuffs.

Swedish researchers, Hedstrom and Isaksson, described outbreaks of acute enteritis that occurred in Sweden from 1946 to 1948. The outbreaks were characterized by diarrhea of adult animals, with a long-lasting decrease in milk secretion and low mortality. The duration of illness in an individual animal was 4 to 5 days, and from 2 to 4 weeks per herd. In retrospect, they concluded that the current outbreaks were a more severe and widespread form of a disease prevalent in regions of Sweden for many years and compared the outbreaks to a disease, recently described by Olafson in New York, called virus diarrhea. But Roberts, who had characterized the acute and the apparent point-source nature of the epidemics and compared the annual incidence of outbreaks seen by Cornell ambulatory clinicians over a 30 year period, suggested that the Swedish disease more closely resembled the diseases described by Jones and Rollinson, and by this time well recognized in Northern US dairies.

Reports on the disease followed in Canada, where the syndrome had been recognized for over 20 years, and Australia, where the syndrome had not previously been recognized. From these early reports to the present, the clinical and epidemiological descriptions of the disease have varied little, and the causal factors have remained elusive.
Eventually WD was recognized as a distinct disease entity, even though there has been no uniformly agreed upon diagnostic criterion. One feature of WD, that appears to be uncontroversial, is that the syndrome includes both cow-level and herd-level definitions. Descriptions of the disease include both the clinical signs of individuals and the appearance of the disease within herds. In 1978, Campbell and Cookingham, in their review of the disease, lamented the lack of complete and accurate descriptive statistics and agreement on the clinical signs. They prepared a composite description of the syndrome from the work of previous researchers:

Winter dysentery is an acute, highly contagious disease of adult cattle characterized by a brief, explosive attack of diarrhea or dysentery. In the northern United States it usually occurs in housed cattle during the winter months and results in a moderate to marked drop in milk production. In affected herds, the attack rate may reach 100% but fatalities are rare. Recovery usually occurs despite treatment, and usually is uneventful. The causative agent is unknown but is presently suspected to be a virus.

This description of the syndrome appears to be commonly accepted among WD researchers, but does not address respiratory signs such as nasolacrimal discharge and coughing, which has frequently been described by others, but is not always present. This description also does not include the presence of blood in the feces as a diagnostic criterion. It is often mentioned that there is blood present in the feces of some affected animals; however, this parameter has not been well quantified. Merriman estimated that blood was present in the feces of at least some animals in about half of the affected herds.
1.2 Pathology

Only limited information on the pathology of WD is available, probably because of the low mortality associated with the disease. Jones and colleagues provided the first pathological description of the disease. They necropsied 4 affected adult animals and found catarrhal inflammation of the jejunum and ileum, characterized by injected serosal vessels, edematous intestinal walls, and a swollen, congested mucosa. Histological examination of the affected intestinal sections revealed a degenerative superficial mucosa overlain with mucus and disintegrated cells. The mucosa was infiltrated with round cells and leukocytes. The vessels of the submucosa were engorged, and the connective tissue edematous. The livers from 3 were friable with engorged gall bladders, characterized histologically by hydropic degeneration of the parenchymal cells. The folds of the abomasum were congested and edematous, and mesenteric lymph glands were enlarged, pale, and juicy. Edwards and Sier euthanized and necropsied 2 severely affected, and recumbent animals. They also found inflamed mucosa of the abomasum, small intestines, and regions of the forestomach. In one cow the mucosa of the ileum was severely inflamed with hemorrhage into the lumen. No lesions were noted in the large intestine. Most dramatic, was the severe inflammation of the upper respiratory tract. Here the mucous membranes from the posterior nasal cavity to the larger bronchii were congested, swollen, petechiated and covered with a sero-fibrinous exudate. The lungs were normal, but there was a bilateral linear parietal pleuritis present following the caudal edges of the third to eighth
ribs. Van Kruiningen later provided pathological descriptions of the lesions in adult cattle, 1 naturally infected and 3 experimentally infected with feces from a WD affected individual. He found similar pathologic lesions in all 4 animals, characterized by reddened abomasal mucosa, segmental hyperemia of the small intestine (especially the jejunum and ileum), a flaccid or dilated small intestine with thin fluid contents, scattered clusters or linear streaks of pinpoint petechial hemorrhage along mucosal ridges of the spiral colon, and scattered parallel linear streaks of petechial hemorrhage in the distal colon. Generally the colons were empty with a slippery, shiny surface and the cecums appeared unaffected. Histological lesions were present in the colons of all 4 individuals, and were characterized by pyknosis, karyorrhexis, granular degeneration, hydropic degeneration and hyaline drop degeneration of clusters of glandular epithelial cells in crypts, midglandular, or near the surface. Along the colonic ridges, engorged capillaries and focal hemorrhages were present in the near-surface laminal propria.

1.3 Treatment

Treatment of the disease is probably not warranted in most instances due to the low mortality and usual spontaneous recovery; however, oral astringents and fluid and electrolytes are sometimes recommended for severely affected animals. Most reports have not demonstrated any efficacious therapy; however, Edwards and Sier may have had exceptional responses to the use of cortisone (50mg. IM) or ACTH (80 IU. IM) since all 8 recumbent animals so treated had recovered within 24 hours. Bull treated over 500 animals from 12 herds with a product containing a nitrofuran, bismuth
subsalylic, zinc phenolsulfonate, and phenylsalicylate as active ingredients. He concluded empirically that treatment of affected animals shortened the duration of illness, though no controlled comparisons were made. In a crude field study, he prophylactically treated 80 of 209 unaffected individuals in 10 affected herds and measured a slightly protective effect which was not statistically significant. In 1957, Roberts summarized his clinical evaluation of various treatments, in vogue at the time, and concluded that none affected the duration of diarrhea in affected animals, nor did prophylactic treatment prevent the onset or severity of disease. He felt that the favorable testimonials attributed to some treatments were actually due to the natural course of the disease, because the most severely affected animals get sick first, milder cases follow and duration of illness is typically short.

1.4 Agents

The infectious, contagious nature of WD seems obvious, and several researchers have demonstrated experimental transmission of disease similar to that described in naturally occurring outbreaks. The first causal investigation of WD was reported by Jones and coworkers in 1931. In a series of experiments they attributed the causal agent of WD to be *Vibrio jejuni* (now *Campylobacter fetus* subspecies *jejuni*), by first infecting calves with the feces from diarrheic cows from WD herds, and isolating vibrio organisms from the diarrheic feces of those calves. Diarrhea was induced in other calves after feeding the cultured organisms. They had only limited success inducing diarrhea in adult animals. From this work, *Vibrio jejuni* was
widely considered the cause of WD for many years. This notion was so popular that 40 years later veterinary textbooks still attributed a causal role for this agent to WD, despite evidence to the contrary.30,31

Hedstrom and Isaksson in 19516, and then MacPherson in 1957,4 suggested that a virus might be responsible for WD. Hedstrom and Isaksson were unable to culture vibrio organisms from the feces of affected animals, but were able to transmit the disease by oral administration of fresh or dried feces but not blood, milk, urine, or tissue emulsions. They were unable to transmit the disease after bacterial (Seitz) filtering. McPherson did transmit the disease to two 18-month-old steers after subcutaneous administration of Seitz filtered fecal filtrates. He was able to serially passage the disease in other animals in this fashion, yet, animals experimentally infected were resistant to subsequent challenge.

Charton, with other French investigators successfully isolated an enterovirus from the feces of cattle from 1 dairy affected with WD.32 The outbreak occurred first in adult cattle then in the young stock. Two heifers developed a painful catarrhal stomatitis and diarrhea 4-6 days after intravenous injection of the isolated virus (WD-42) passaged 8 times in bovine kidney cells.

Scott and others investigated 20 New York dairies with WD and reported the results of thier etiologic study in 1973.26 They were unable to identify Vibrio jejuni, or Salmonella in any affected animals. They searched for bacteria, viruses, mycoplasma, and protozoa in the feces of affected animals without finding a causative agent. They
did substantiate the infectious nature of the disease by experimentally recreating the
disease in several 2-year-old heifers after oral or oral-nasal inoculation with feces or
intestinal contents. The incubation periods were 3 to 5 days. Scott and Anderson
demonstrated, in 1976, that these outbreaks were not serologically related to the WD-
42 enterovirus isolated in France.\textsuperscript{33}

In 1975, Homer, Hunter, and Kirkbride reported finding a coronavirus-like
agent by electron-microscopy in the feces of cows from a New Zealand dairy herd
experiencing an outbreak of diarrhea among 14 of 100 adult animals. No bacterial
pathogens had been identified from the outbreak.\textsuperscript{34} In 1979, Durham, Stevenson, and
Farquharson summarized the rotavirus and coronavirus diagnostic laboratory findings
from diarrheic feces of many species in New Zealand.\textsuperscript{35} They reported that 14 of 29
diagnostic submissions where coronavirus was found in cattle came from animals >1
year of age.

Similar reports incriminating coronavirus-like agents in WD followed, as
diagnostic methods in virology became more sophisticated. In 1980 Takahashi and
colleagues, from Japan, reported finding coronavirus-like agents by electron-
microscopy in the feces of adult cattle from 10 herds affected with epizootic diarrhea,
and were able to isolate the virus in cell culture.\textsuperscript{36} High prevalence of bovine
coronavirus (BCV) hemaglutination-inhibition sero-responses were found in paired sera
from those herds.
Espinasse and coworkers reported, in 1982, finding a corona-like agent in the feces of cattle from 6 beef and dairy herds in France affected with winter dysentery. They were unable to demonstrate vibrions, *Salmonella* spp., or spirochetes in the feces, and serologic responses to infectious bovine rhinotracheitis virus (IBRV), bovine viral diarrhea virus (BVDV), parainfluenza 3 virus (PI-3), adenovirus type 3, reovirus 3, bovine respiratory syncitial virus or *Chlamydia psittaci* were inconsequential.

Van Kruiningen and coworkers reported, in 1985, their investigation of 2 Connecticut dairies with WD. They conducted transmission studies, pathology studies, and looked for evidence of infectious agents. An experimental herd of six 2 ½- to 3-year-old, pregnant Holstein cows were exposed through their feed and water to fresh feces obtained from diarrheic animals from the first outbreak. On the third post-inoculation day 2 animals became febrile (39.8 C and 40.4 C) and on the fifth day, 5 animals had profuse, brown fluid diarrhea. An experimental herd of 8 animals were exposed to feces from the second outbreak, but none of these individuals became ill. No viral particles were observed by electron microscopy, or immune electron microscopy (IEM). Vibrions (*Campylobacter* spp.), *Salmonella* spp., *Shigella* spp., and *Yersinea* spp. were not cultured except *Campylobacter fetus* subsp. *jejuni* and a *Shigella* sp. was cultured from the 1 animal that died during an outbreak. They concluded that histological lesions suggested viral injury, but that inopportune sample timing may have made viral detection unachievable. Two years later, in light of other evidence incriminating BCV in WD outbreaks, these investigators re-examined tissue
sections from this prior investigation and detected coronavirus-like organisms within crypt epithelial cells in the spiral colon; and by using immunohistochemistry, found positive reactions in degenerate and intact crypt epithelial cells, and macrophages. They considered the lesions of WD comparable to the lesions found in BCV associated neonatal calf enteritis.28

In 1988, Saif and colleagues reported their investigation of a WD outbreak in an Ohio research dairy.20 This outbreak occurred over a 3 week period and involved 20 lactating animals of a herd of 136. No enterotoxigenic Escherichia coli, Salmonella spp., Cryptosporidia spp., or coccidia were found in the feces of affected animals. There was also no evidence of BVDV infection found using indirect immunofluorescence after inoculation of feces or buffy coat specimens onto secondary bovine turbinate cell monolayers. They did find coronavirus particles in the feces using IEM and protein A gold IEM using NCDV coronavirus specific antiserum. In contrast, coronavirus was not found in the feces of non-diarrheic herdmates.

One dairy and one beef herd in Canada with signs attributable to WD were investigated in 1989 by Durham and colleagues.19 All ages of cattle were affected in the dairy, and adult cows, and yearling heifers were affected in the beef herd. Coronavirus particles were identified by electron microscopy in all ten fecal samples from the dairy and 2 of 3 fecal samples from the beef herd; all three fecal samples from the beef herd were positive for coronavirus by fluorescent antibody test. Some campylobacter-like organisms were visualized in some fecal smears from both herds, but no
Campylobacter or Salmonella organisms were isolated by culture of 10 fecal samples from the dairy, and Campylobacter was isolated from 1 of 3 fecal samples examined from the beef herd. There was no serological evidence of infection by IBRV, BVDV, or PI-3 virus.

Fleetwood and colleagues reported, in 1989, the results of their search for BCV from 9 English herds with WD. They examined fecal samples from affected animals by ELISA and electron microscopy and found BCV in 3 of the herds. In one herd, feces was examined for Salmonella spp., Campylobacter spp., coccidia, cryptosporidia, acid-fast organisms, and rotavirus; all were negative. Feces were positive for BCV by ELISA from 3 of 6 affected cows and 2 of 2 affected heifers. Latex agglutination inhibition seroresponses were detected in these same individuals.

In 1990, Saif and Benfield reported the successful cell-culture propagation of a coronavirus isolated from WD affected individuals after passage through gnotobiotic or colostrum deprived calves. The isolates were morphologically and antigenically similar to the Mebus NCDV strain of BCV from neonatal calves.

A prospective longitudinal investigation of risk factors for WD in 15 French herds was reported in 1990 by Jactel and colleagues. Six of 36 WD affected individuals from these herds were positive for BCV by ELISA; no Campylobacter spp. were isolated, but Proteus (4), Bacillus (13), Streptococcus (10), Pseudomonas (2), and Escherichia (2) were isolated from some of the 34 fecal samples cultured.
Saif and colleagues, in 1991, compared the diagnostic findings from samples submitted from 6 WD herds (characterized by adults cows with hemorrhagic diarrhea) with the findings from 3 herds affected with non-hemorrhagic diarrhea and not considered WD. Coronavirus was detected by IEM in all 6 WD herds, but was not detected in the other diarrheic herds. Within the WD herds there was a higher prevalence of affected individuals with BCV particles in the feces compared to unaffected herdmates. Plaque reduction neutralizing antibody seroresponses (>4-fold rise in neutralizing antibody titer) were detected in all 6 WD affected herds, but in none of the non-WD affected herds. Group B rotavirus particles were identified in the feces of 2 of 2 diarrheic individuals from a non-WD herd. No evidence of BVDV antibody seroresponse was found in any of the herds by indirect immunofluorescence.

Alenius with others reported, in 1991, on a serologic investigation of 9 WD affected dairy herds in Sweden. Following the outbreaks, animals in all 9 herds demonstrated seroresponses to BCV and 1 herd demonstrated seroresponses to BVDV. Interestingly, the herd with seroresponse to BVDV had individuals with high acute titers to BCV, while the cows in the remaining 8 herds had negative acute BCV titers. In the same report, the investigators noted the BCV seroprevalence among heifers in 58 herds. The authors reported that 35% of the herds had low prevalence of individuals with titers against BCV and 50% had high prevalence, but failed to report if any herds were entirely BCV seronegative.
In 1991, Van Kruiningen and colleagues also reported the results of a serologic investigation of 8 WD affected herds in New England and New York. Practicing veterinarians from New York, Massachusetts and Connecticut were invited to submit acute and convalescent serum samples from 5-6 individuals in WD affected herds. Submissions with late acute sampling, or very long convalescent periods were not included in the study. All 8 WD affected herds meeting the criterion for inclusion in the study had animals demonstrating ≥4-fold BCV antibody ELISA titer increases.

Koopmans, with other researcher from the Netherlands reported, in 1991, an association between Bredavirus infection in young calves and diarrhea, and Bredavirus infection of adult cattle in herds affected with WD. In this controlled field investigation, the prevalence of seroreponse to BCV and Bredavirus among adult animals was compared between 19 WD case herds and 8 unaffected control herds sampled during the same time period. The odds of Bredavirus infection were significantly greater among individuals of WD affected herds compared to controls herds. The odds of coronavirus infection was not significantly different between the 2 groups.

In 1992, Connecticut and Dutch researchers together analyzed serum samples from New York and New England WD affected herds for evidence of Bredavirus and coronavirus exposure. The serum samples came from previously examined and newly acquired submissions. At least 4-fold seroresponses to BCV were demonstrated
in 22 of 36 (61%) individuals from 8 herds, while only 3 of 37 (8%) demonstrated a Bredavirus seroresponse.

Carmon and Hazlett, in 1992, found evidence of BCV infection from diagnostic samples submitted to the Ontario veterinary diagnostic laboratory from an unknown number of WD herds in Canada. Three assays were used for diagnosis: an antigen detection ELISA, a direct immunofluorescence assay, and a BCV virus neutralization assay.

Camero, with others, in 1992, demonstrated evidence of BCV infection in each of 8 herds affected with WD in France. Particles of BCV were identified in the feces of affected individuals by IEM. Isolates in cell culture were demonstrated to be similar to calf strains of BCV by seroneutralization and direct immunofluorescence.

Diagnostic submissions from WD affected individuals in Quebec were used in a 1994 study by Athanassious and colleagues to compare several diagnostic techniques. Of 52 WD submissions from an unknown number of herds, 25 were identified as coronavirus by direct electron-microscopy, 24 were positive by ELISA and 20 positive by both methods. Seventeen of 21 samples were positive for coronavirus by both PAG-IEM and ELISA. Some corona-like particles by electron microscopy were not labeled by PAG-IEM suggesting that these were antigenically different from other coronaviruses, or were other viruses, perhaps torovirus-like. The authors suggested that other viruses may also play a causal role in WD.
Since 1975 evidence that BCV may be causally associated with WD has mounted. Still evidence of coronavirus infection has not been demonstrated in all WD herds which have been examined\textsuperscript{39}, and some researchers have reported finding other viral agents from affected herds.\textsuperscript{24,32,45} It is not surprising that BCV infection has not been demonstrated consistently: the virus is apparently shed early in the course of the disease, shedding is transient,\textsuperscript{16,22} and BCV is difficult to propagate in cell culture.\textsuperscript{15} Regardless, the evidence suggesting that BCV is causally associated with WD outbreaks is circumstantial, coming from investigations conducted within WD affected herds, and often lacking controlled comparisons. Since some healthy, non-diarrheic, adult cattle also shed BCV in their feces,\textsuperscript{46-49} baseline investigations on BCV infection of normal herds are necessary before we can fairly assess the evidence for causality in WD.\textsuperscript{17} Biological plausibility, which supports the hypothesis that BCV is causally associated with WD, comes from the histological similarity between lesions from calf coronavirus infections and cows with WD,\textsuperscript{38} experimental reproduction of diarrhea in adult cows after exposure to a calf infected with a WD isolate of BCV,\textsuperscript{50} and the tropism that BCV demonstrates for both respiratory and intestinal epithelium which could explain the digestive and respiratory components of the disease.\textsuperscript{15}

In light of the evidence that BCV may be causally associated with WD, it is interesting to consider the comments of MacPherson.\textsuperscript{4} In 1957, he considered WD in cattle to be the epidemiological analogue of the common cold of humans. The basis of MacPherson's comparison was the similarity of the 2 diseases in transient immunity,
high morbidity, and tendency for recurrent attacks particularly during the winter months. More recently, human coronavirus 229 E, and the more closely BCV related human coronavirus OC43, have been associated with the common cold in humans.\textsuperscript{51}

It is not clear that WD is caused by a singular agent. Previous researchers have speculated on the possibility that WD may not be a distinct disease, but the manifestation of several diseases that are difficult to distinguish clinically, such as bovine viral diarrhea (BVD) or salmonellosis.\textsuperscript{3,14} In the past, some confusion has probably existed in distinguishing WD from other bovine viral diseases.\textsuperscript{14} In field investigations, failure to clearly differentiate disease entities can lead to wrong conclusions, due to biases in measures of association.\textsuperscript{52} Etiologic studies of WD probably have been hampered by lack of clearly defined diagnostic features for WD.\textsuperscript{3} Other diseases of cattle, such as nutritional enteritis, acute BVDV infection, rinderpest, parasitism, and salmonellosis, have similar clinical signs and must be distinguished from WD by consideration of herd history and diagnostic testing\textsuperscript{14} leaving us to wonder how sure we can be that WD is attributable to a singular agent when currently the disease is diagnosed by the exclusion of other similar known diseases.

Perhaps the disease most similar to WD in cattle is acute BVDV infection. The first outbreaks of viral diarrhea, later attributed to BVDV, were described by Olafson and colleagues in New York during 1946.\textsuperscript{53} Olafson and colleagues initially distinguished the 2 syndromes primarily by relative severity and for some time afterwards there was confusion among researchers regarding the differentiation of
BVD and WD. Some WD outbreaks were compared to both diseases.\textsuperscript{4,6} McPherson differentiated WD outbreaks from BVD, based on the clinical signs of individuals: BVD infection characterized by the presence of oral ulceration, and WD by the presence of blood in the feces.\textsuperscript{4} However, oral lesions are not always present in outbreaks of BVD,\textsuperscript{4} and have been seen in WD outbreaks.\textsuperscript{19} Using data from a vaccine trial, Kahrs was unable to demonstrate a serological relationship between BVDV or IBRV and WD; and vaccination against these agents was not protective against WD outbreaks.\textsuperscript{3} It is not yet clear that BVD and WD can be distinguished clinically in every case, since BVD, which is the acute infection of BVDV, is now known to range widely in severity, with subclinical infections the most common.\textsuperscript{54} As previously mentioned, the role of BVDV in a WD outbreak in Sweden was recently suggested.\textsuperscript{42}

1.5 Host factors

Throughout the search for causal understanding of WD, many researchers have commented on the role of immunity and the occurrence of the disease. In this paper the term immunity will refer to both the innate and the specifically acquired resistance, or lack of susceptibility, to disease.\textsuperscript{55,56} Immunity to infectious diseases can be considered as characteristic of the individual, and the herd.\textsuperscript{56}

It is not clear if there is any innate, or acquired age-related immunity to WD. Steffen described a disease of cattle of all ages;\textsuperscript{1} however, Jones and Little\textsuperscript{2} and later Roberts\textsuperscript{12} and MacPherson\textsuperscript{4} suggested that young calves were less likely to be affected by WD.\textsuperscript{2} MacPherson suggested that resistance in young calves may have been due to
protection from maternal antibodies or because of reduced stressors among that group of animals. Some researchers, starting with Jones, have induced diarrhea in young conventionally raised calves or colostrum deprived and gnotobiotic calves during WD transmission studies. From a farmer survey in Sweden, Traven and colleagues, reported that 31% of the WD affected herds reported scour in young calves. Roberts suggested that the most susceptible cattle are the young, 2- to 6-year-old cows, that had recently freshed and were in heavy lactation. Others have described similar findings. Aged cattle also appear to be more resistant to disease. Edwards and Sier suggested that recent stressors such as parturition, or debility as from poor nutrition, increased the severity of disease in individuals. One severely affected beef cow from a Colorado herd experiencing WD, had depressed hepatic copper levels, and responded following copper EDTA injections; the remainder of the herd recovered without therapy.

Specific immunity apparently develops following disease. In 1 of the first descriptions of the disease, Steffen reported that recovered animals appear to be immune indefinitely. However, Jones commented that, while a single outbreak in a year was common, some practitioners had observed more frequent attacks. MacPherson observed that individuals could be clinically affected up to 5 times before becoming resistant to WD, but in no case did they observe disease in the same animal within a 10 month period. However, Hedstrom and Isaksson described outbreaks within some herds that recurred 2-3 months after the first, and suggested that
recurrence in individuals was quite common. MacPherson provided experimental evidence of post-exposure immunity when he was unable to induce disease, following rechallenge with filtered virus, in 2 steers that had been previously infected. El-Kanawati and colleagues demonstrated cross-protective immunity in gnotobiotic calves infected, and then cross-challenged with heterologous BCV isolates from both WD and neonatal calf diarrhea.

Herd immunity may also be a factor influencing the occurrence of WD outbreaks. MacPherson noted that herds, and whole regions, experienced the disease nearly simultaneously. But, while Edwards found the same pattern of disease occurrence in Australia, he also noted that some herds within affected regions avoided disease. Roberts retrospectively demonstrated a cyclical pattern of high and low annual WD incidence in New York herds, which he felt indicated years of protective herd immunity following years of high incidence. He also demonstrated that the herds with the highest attack rates had not experienced WD within the past 3–4 years, and thus may have had lower herd immunity. Traven and colleagues found that Swedish herds affected with WD in the previous 4 years had lower average disease severity scores, which they felt suggested greater herd immunity among those herds more recently affected. However, in a prospective study of farmer diagnosed WD in New York, White and colleagues found increased risk for WD among those herds that had previously experienced the disease. The existence of carrier animals in herds
previously experiencing WD could explain why those herds are at greater risk for future outbreaks.\textsuperscript{4,59}

Alenius, and colleagues, found that 8 of 9 WD affected herds they investigated were seronegative to BCV at the time of their outbreak, but then seroconverted, suggesting low herd immunity to that agent. They proposed that herd immunity is initially high following a WD outbreak, but as older animals are replaced by immunologically naive young-stock, herd immunity is reduced, until the herd again becomes susceptible.\textsuperscript{42} Other studies have demonstrated BCV seroresponses within WD affected herds;\textsuperscript{23,41} however, to date, no study has clearly demonstrated that herds with low specific immunity to BCV, or any other virus, are at greater risk for WD.\textsuperscript{15}

1.6 Environmental factors

That the environment influences the occurrence of disease is one of the oldest concepts of medicine. While often causal research focuses specifically on infectious agents, some epidemiologists consider that agent exposure is only one component of a myriad of environmental factors. Certainly it is the environment where host and agent meet, and reasonably, environmental factors influence agent and host interactions.\textsuperscript{60} Even the name WD suggests that environmental factors contribute to the occurrence of the disease.

Steffens suggested that the disease was most prevalent in the winter months.\textsuperscript{1} As the accepted name, WD, implies, this point is not in contention. However, there has been debate over what specific winter conditions increase the risk for WD and whether
the disease does in fact occur during the summer months as well. Recently, Traven and colleagues reported the monthly incidence of farmer diagnosed WD in Sweden during a 1 year period. While the incidence was higher from October to March, peaking in December, outbreaks were reported during every month except July. Jones, with others, suggested that the disease might occur in individuals during the summer months but with less severe clinical signs so as to be practically unnoticed.

The reason for the seasonal nature of the disease is not clear. Some researchers have suggested that closed housing is used more commonly during the colder months, and may be a factor encouraging the transmission of infectious agents because of closer confinement of cattle, and poor ventilation. Merriman, a Michigan practitioner, had not observed the disease in pastured cattle. Close confinement and poor ventilation would facilitate transmission of agents spread by aerosol such as BCV or BVDV. Bull, a New York veterinary practitioner, credited chilling drafts, or the consumption of icy water or ensilage with reducing the resistance of individuals to WD. Changes in the weather have been noted by some to be a predisposing factor. MacPherson observed that outbreaks occur coincident with sudden drops in atmospheric temperature. In Australia, Hutchins with others, noted outbreaks coinciding with cyclonic weather changes in June, and Edwards and Sier noticed that the spread of WD between herds and the morbidity within herds decreased rapidly as ambient temperatures increased. In France, Jactel with others, found that WD outbreaks tended to be preceded by temperature drops in the barns of at least 1.7°C.
followed by a rise in temperature. They also found stronger associations to disease with air temperatures <9°C in the barns and feed and water temperatures <5.4°C and <6.5°C, respectively. They suggested that exposure to cold feedstuffs and lowered ambient temperatures might induce a thermal stress which could reduce an animal's resistance to the disease.\textsuperscript{22} Cooler winter temperatures may facilitate the effective transmission of WD infectious agents by providing a more favorable environment for their survival. For example, coronaviruses survive best at low temperatures and low ultraviolet light intensity more typical of winter months.\textsuperscript{59}

Because of the infectious, contagious nature of this disease, it is not surprising that many researchers have noted an association between the introduction of new cattle, or visits from other farmers, veterinarians, feed salesmen, \textit{et cetera} and the occurrence of WD outbreaks.\textsuperscript{1,6,8,11,12,18} The effect of herd size on WD occurrence is not clear. Some researchers have demonstrated increased risk for WD among larger (>60 cows) herds,\textsuperscript{21,58} while others have found that housing animals with too much or too little space, regardless of herd size, increased the risk for WD.\textsuperscript{22}

Confusion exists about the role of feed changes and WD outbreaks. Merriman conjectured in 1953 that feed changes were a predisposing factor in the majority of cases.\textsuperscript{8} He proposed 2 possible mechanisms of action by which feed changes could increase the likelihood of an outbreak: 1) mechanical introduction of the infectious agent, or 2) enhancement of the virulence of an already present agent in the wake of
low-grade indigestion. Others researchers have found no reason to suspect feed changes to be related to WD occurrence.5,11,12

Several researchers have described regional spreading of WD outbreaks,4,6,7,11,13 suggesting that herds in the vicinity of an outbreak may be at greater risk for the disease. This hypothesis was supported in by the findings of White, Shukken and Tanksley in 1989.58 They conducted a prospective longitudinal study of farmer diagnosed WD from 113 New York dairies, and demonstrated a time-space clustering of the outbreaks.

1.7 Future research

It is clear from this discussion that many insightful researchers have contributed to our understanding of WD, but have been frustrated in their attempts to understand the causal of this disease. Winter dysentery is recognized as a rapidly contagious infectious disease with some specific clinical and epidemiologic characteristics, yet after at least 65 years of research, questions remain concerning whether or not a single infectious agent is causally associated with WD, or if more than one agent acting alone, or in combination with other agents could be responsible for the same collection of clinical signs collectively identified as the WD syndrome. With advances in diagnostic virology, we have accumulated considerable evidence that BCV may be causally associated with some WD outbreaks, although the agent has also been demonstrated in normal feces. It is not clear if BCV may be playing the role of a causal agent, an opportunistic invader, or or part of the normal intestinal microflora.59 In order to be
fruitful, future investigations of WD must utilize epidemiologically sound study designs, and advanced diagnostic methods, which incorporate controlled comparisons. Host, agent, and environmental risk factors have been suggested, and future causal hypotheses must consider each of these. Further, since the occurrence of WD has been demonstrated to have geographical, herd-level, and individual-level risk factors, future investigations will need to test causal hypotheses at appropriate levels of investigation.
1.8 References


A REVIEW OF BOVINE CORONAVIRUS, WITH DISCUSSION OF POSSIBLE MECHANISMS OF VIRUS SURVIVAL AND DISEASE PROPAGATION WITH PARTICULAR REFERENCE TO WINTER DYSENTERY

The coronaviruses are enveloped single-stranded RNA viruses with a wide range of mammalian and avian hosts including humans. Coronavirus infection is associated with a wide range of diseases including respiratory and enteric diseases, encephalomyelitis, hepatitis, serositis and vasculitis. In most cases coronaviruses replicate in respiratory tissues and transmission of the virus is usually by this route, although often the virus is shed in the feces as well.\(^1\) Coronavirus is one of several viruses considered agents of the common cold in humans. In most cases, coronavirus infection is associated with severe disease in newborn and young animals, but mild disease or inapparent infections in adults.\(^2\)

The coronaviruses are so named because of their characteristic physical resemblance to a solar corona, due to the large club shaped peplomers that project from their envelope when viewed by electron microscopy. The virion is pleomorphic, though generally circular, and ranges from 75 to 160 nm in diameter (average diameter 100 nm). The envelope is composed of a lipid bilayer derived from host intracellular
membranes, and three structural glycoproteins, M, HE (absent in some), and S. The long, flexible, helical nucleocapsid phosphoprotein, N, is another structural protein of the virus intimately associated with the viral genome.\(^2\)

The single genus, *Coronavirus*, is classified into four antigenic groups, two subgroups of avian and two subgroups of non-avian viruses.\(^2,3\) Antigenic group I (mammalian) includes human coronavirus 229E, transmissible gastroenteritis virus of swine, feline infectious peritonitis virus of cats, and canine coronavirus. Group II (mammalian) includes human coronavirus OC43, mouse hepatitis virus (many serotypes), bovine coronavirus, and porcine hemagglutinating encephalomyelitis virus. Group III (avian) includes at least eight serotypes of infectious bronchitis virus of chickens. Group IV (avian) includes the bluecomb disease virus of turkeys.\(^2\) The coronavirus of turkeys (TCV) has been shown to be, on a molecular basis, almost undistinguishable from bovine coronavirus (BCV) and it has been proposed that they should be reclassified.\(^3\)

### 2.1 Bovine coronavirus

The characteristics and related diseases of BCV were recently reviewed by Clark.\(^1\) Virus particles are round and pleomorphic, varying in diameter from 80 to 160 nm, with a mean diameter of 120 nm. The viral particles possess 4 major structural proteins, N, M, S, and HE, and a number of minor structural proteins.\(^4\)

Bovine coronavirus infection is most commonly diagnosed by examination of feces or nasal secretions for the presence of virus, or by demonstration of rising serum
antibody titers. The most commonly applied virus detection methods include electron microscopy (EM), immune electron microscopy (IEM), ELISA, fluorescent antibody assays, and hemagglutination.\textsuperscript{5-12} The most common serum antibody assays to diagnose and characterize immunological responses to BCV include serum neutralization, hemagglutination inhibition, and ELISA.\textsuperscript{12-14}

Bovine coronavirus is an important agent of neonatal calf diarrhea (NCD) and has been implicated in other disease syndromes as well.\textsuperscript{15-18} The virus was demonstrated and described as an agent of calf diarrhea in the United States; first by EM in 1972, and then isolated in cell culture a year later.\textsuperscript{19,20} Since then the virus has been recognized world-wide.\textsuperscript{2} The disease is commonly seen in calves at about 1 to 2 weeks of age and the disease typically lasts for 4 to 5 days. The virus is transmitted by the oral and respiratory routes and infection leads to diarrhea within 24-30 hours.\textsuperscript{21-23} The disease is characterized as an acute infection, leading to destruction of the villus epithelium of the small, and mucosal erosions in the large intestines.\textsuperscript{2,24} Damage to the intestinal villi leads to a malabsorptive diarrhea and in some cases the severe losses of water and ions may result in death.\textsuperscript{24} The virus also replicates in nasal epithelial cells and this may provide a mechanism for rapid spread of the virus to other susceptible hosts,\textsuperscript{17,25} or cause outright respiratory disease.\textsuperscript{23,26-28} As discussed in the introduction to this dissertation, a large body of evidence from around the world has implicated BCV in outbreaks of winter dysentery (WD) in adult cattle.
Currently, the only animal models for BCV enteric infection are gnotobiotic (GN) and colostrum-deprived (CD) calves. Experimental infection of a number of laboratory animals has been unsuccessful; however, Tsunemitsu recently isolated a coronavirus, undistinguishable from BCV, from a number of captive non-domesticated ruminants affected by an outbreak of bloody diarrhea and also found coronavirus seropositive wild deer in Wyoming and Ohio.

It is not clear if the BCV isolated from various disease syndromes represent distinct groups. The known neonatal calf diarrhea (NCD) BCV isolates belong to a single serogroup, and in vivo cross protection has been demonstrated in gnotobiotic and colostrum deprived calves between heterologous NCD and WD BCV isolates. However, some differences have been reported in the HI activity between NCD and WD BCV. Genomic differences between different strains of BCV have also been detected; however, it is not yet clear if these differences are distinctive between the disease syndromes of NCD, respiratory disease, and WD.

Even though BCV is sensitive to environmental conditions, it is not uncommon for BCV infections and the associated disease in neonatal calves to occur year after year on some beef and dairy farms. On most beef cow-calf operations calving is seasonal, mostly in the early spring in the US, yet some farms experience BCV diarrhea with each calving season. As described above, these calf scours epidemics appear to be acute and self-limiting. How BCV is maintained between host infections and between calving seasons is an enigma, but could be explained by subclinical, persistent or
recurrent infections of older animals. Similarly, if BCV is in fact a causal agent of WD, then the mechanism of virus survival from season to season is unknown, but since herds previously experiencing seasonal outbreaks of WD appear to be at greater risk for new outbreaks of the disease, a reservoir of infection on those farms is possible.

2.2 A review of the mechanisms of virus survival and persistent viral infection

It is important to understand the strategies employed by viruses to survive and the known mechanisms of persistent viral infection in order to evaluate whether these mechanisms might be important in the propagation of BCV related diseases. Virus survival depends on their ability to replicate in host cells, be shed from those infected cells and then invade and infect the cells of a new host. The strategies used for viral replication are varied. In general these strategies lead to either acute infections, typically leading to illness or death of the host, or persistent infections where disease may be weak, delayed or subclinical. Successful survival strategies for acute viral infections require that the virus have the ability to replicate rapidly and then quickly be disseminated, usually in large numbers, to new susceptible hosts. Because of the damage they often do to their host, viruses surviving by acute infection are forced to continually seek new cells or hosts to infect. These types of acute infections are among the best known by clinicians, pathologists and microbiologists because they cause diseases of a defined clinical picture and characteristic pathology. However, viruses that can persist in the host are ultimately successful because of their abilities to survive within the cells that provide their sustenance and avoid the hosts immune system. A
successful persistent infection strategy would necessitate the absence of the cell lysis and inflammatory signs we commonly associate with acute viral infections.40

2.3 Viral mechanisms of persistent infection

Persistent viral infections are of importance for 4 reasons: 1) they are of epidemiologic importance by the creation of carrier animals that can serve as sources of infection for other animals, allowing the virus to persist in the population; 2) they may be reactivated and cause recurrent acute disease episodes; 3) they may lead to immunopathologies; and 4) they are sometimes neoplasia associated.39

Persistent infections are classified into three categories (latent, chronic, slow) relative to the extent of replication occurring during the period of persistence. In latent infections, virus is not evident except when reactivation occurs. With chronic infections, virus is always demonstrable often with shedding. Disease may be chronic, absent, or delayed, often with an immunopathologic consequence. Slow infections are characterized by gradual increases in infectious virus during a long preclinical phase, leading to slowly progressive lethal disease.39

The mechanisms for persistent infection relate to the strategies of the virus to bypass the host defenses that would eliminate virus in acute infections. Primarily these strategies relate to factors of the virus, sometimes to factors of modification of the host defenses, and in some instances the two factors interact. Virus related factors include integration of the viral genome into the host DNA, non-immunogenicity of the agent,
growth in protected sites, and avoidance of the host immune defenses by antigenic drift.\textsuperscript{39}

Host defense mechanisms may be altered by viral infection of host immune system cells leading to induction of non-neutralizing antibodies, defective cell-mediated immunity, or impairment of antigen presentation from growth in macrophages.\textsuperscript{39} For example it has been shown with lymphocytic choriomeningitis virus infection in vivo that viral clearance is primarily associated with the activity of virus-specific cytotoxic T lymphocytes restricted by H-2 molecules on the mouse major histocompatibility complex (MHC). When these cells fail to generate, or become depleted, infection progresses from acute to persistent.\textsuperscript{41}

In summary, the general strategies of viral persistence are to: 1) remove recognition molecules on infected cells by altering the expression of viral protein, altering the expression of MHC molecules, or altering the expression of adhesion molecules; 2) interfere with immune cell function leading to immunosuppression; 3) hide in cells lacking MHC expression (neurons); 4) generate variants that escape antibody and cell-mediated immunity recognition during the nonlytic phase of viral replication; 5) generate mutants of variants; or 6) diminish expression of viral genes or their products.\textsuperscript{40}

\textbf{2.4 Mechanisms for persistent infection of coronaviruses}

Coronavirus infections in some animal species have been shown to involve chronic or persistent infection. Some coronaviruses readily establish persistent
infections in tissue culture and in animals; these persistent infections often lead to subacute or chronic diseases in animals. In fact coronaviruses show a greater tendency to establish persistent infection in tissue culture compared to other positive stranded viruses. Chronic or persistent infection has been demonstrated to be important in the epidemiology of FIP infection in cats, avian infectious bronchitis, and murine hepatitis virus infection in mice.

Early suggested mechanisms for persistent coronavirus infections included the integration of virus sequences into the host-cell DNA, and episome formation, establishment of attenuated temperature sensitive (ts) or poorly growing mutants, interfering virus, and interferon production. These mechanisms are still under investigation and new mechanisms have been proposed. The following studies of both in vitro and in vivo host systems help to illustrate at least some of the strategies employed by coronaviruses to maintain persistent infections.

2.5 Mouse hepatitis virus as a model of coronavirus persistent infection

Murine coronaviruses are able to produce infections that are acute or persistent in nature. Murine hepatitis virus (MHV) is the most well studied model of persistent infection, both in vitro and in vivo, of the coronaviruses. MHV causes many diseases in susceptible rodents including gastroenteritis, hepatitis, and neurological diseases.

Early evidence that virus could be isolated from latently infected cells in vitro was found when a cold sensitive MHV mutant was rescued from latently infected mouse neuroblastoma cells by cell fusion to permissive cells.
A recent *in vitro* study provides evidence for viral mutation as a mechanism for the development of persistent infection of coronaviruses. A mutant virus isolated from Ki-BALB cells persistently infected with the MHV-S strain of murine coronavirus was compared to the parental virus. The mutant virus, MHV-S no. 8, showed cold sensitivity to the formation of polykaryocytes and lacked the HE protein. Differences in the genomic structure between the mutant and parental virus were found in the addition of 111 nucleotides (nt) inserted just upstream of the intergenic consensus sequence preceding the N gene. This nucleotide insertion between genes M and N resulted in two consensus sequences separated by 111 nt. Sequencing data showed that both consensus sequences were used for mRNA synthesis, the upstream intergenic consensus sequence demonstrated reduced transcriptional activity. The altered genomic structure of the mutant virus probably underwent non-homologous RNA-RNA recombination between genomic and subgenomic RNA species.45

Earlier, other researchers found that host-cell factors were responsible for *in vitro* persistent MHV infection in a subline of mouse LM fibroblasts. In this tissue-culture cell line, persistence was due to the ability of the host cell to withstand the cytopathic effects of the virus rather than involving any apparent change in the input of the wild type virus.46

Evidence is also accumulating relative to mechanisms of *in vivo* coronavirus persistent infection. Different inbred strains of mice have been investigated relative to the outcome of acute or persistent infection with MHV. Resistance of mice to acute
infection is inherited by recessive genetic traits for different viral strains. The expression of genetic resistance is at the interaction of the mature macrophage with the virus. Virus replication in macrophages of resistant mice is restricted while virus replicates well in macrophages of susceptible mice. Other mouse strain related factors of resistance are expressed at the target cell type. Infections can also be modulated by T cell lymphokines and interferons, macrophage immunomodulatory secretions and by corticosteroid hormones. Thus cell-mediated immune functions may be impaired or enhanced by chronic infection.

Suckling mice inoculated with MHV invariably develop fatal acute encephalitis, however this outcome can be prevented if they receive an infusion of protective antibody, or if they are nursed by immunized dams. Suckling mice thus protected from acute MHV infection may later develop a hindlimb paralysis due to persistently infected neural cell MHV infection. Virus isolated from the brains and spinal cord of hindlimb paralyzed mice appeared identical to the initial infecting strain of virus clinically and biochemically. In this case it appeared that the inability of the mouse immune system to eradicate the infection was important in the pathogenesis of MHV persistence. The tropism of MHV for neural cells undoubtedly aids the virus in its development of persistent infection, due to the unique immune response mechanisms that occur within the central nervous system (CNS). Cell mediated immunity appears to play a major role in the immune response to MHV infection of the CNS but fails to eliminate the infection resulting in persistence.
2.6 Persistence of coronaviruses in other host systems

Persistent infection by human coronavirus 229 E has been established in human tissue culture cells, shedding high titers of infectious virus continuously, even after 300 passages over two years. The infected cells were resistant to homologous superinfection, but supported normal titers of polio virus replication. Temperature appeared to play a role in establishing and maintaining the persistently infected cell cultures, suggesting a temperature sensitive mutation.

Recently, by the use of polymerase chain reaction to follow the structure of the common 5' -terminal sequence of bovine coronavirus subgenomic mRNA throughout the establishment of persistent infection in human rectal tumor cell culture, it was discovered that a change occurred in the leader sequence that is postulated to be causally related to the viral persistence. The selection of an intraleader open reading frame apparently resulted in a general attenuation of mRNA translation which was followed by a consequent attenuation of viral replication. This may be one mechanism by which coronaviruses, and possibly other RNA viruses with a similar transcriptional strategy, maintain a persistent infection.

2.7 Proposed mechanisms of persistent infection of bovine coronavirus

If BCV persistence does occur in natural infections, it is likely that the mechanisms are similar to those described for other coronaviruses. Studies of MHV in vitro and in vivo, and human and bovine coronavirus in vitro, suggest that several mechanisms exist whereby coronaviruses may induce persistent infection. It appears
that the critical factor is that the host cell is not killed by the infection so that the host-parasite relationship continues to function. The survival of the cell in spite of viral infections may occur by viral factors of attenuation through some form of mutation, or by alterations of host factors of resistance. It is not unreasonable to expect that other coronaviruses may employ similar, as yet undetected, strategies for persistent infection. Persistent viral infection may explain some puzzling aspects concerning the epidemiology of naturally occurring bovine coronavirus infections such as NCD and WD.

Some evidence suggests that persistent infection may be a mechanism of survival used by BCV. In one report, BCV viral antigen was shed for three days following acute infection of 5-day-old colostrum-deprived (CD) calves, but was detected in crypt cells and Peyers patches for at least three weeks following infection in some calves. Chronic excretion of BCV, detected as viral antigen or as antigen-antibody complexes, by normal adult cows has been reported. One of these studies found an increase in the prevalence of virus shedding at the time of parturition among non-vaccinated adult cows compared to vaccinated adult animals, though both groups were found to shed virus. Increased viral shedding at the time of parturition, perhaps due to hormonally mediated suppression of immunity, would seem an excellent viral strategy to assure sufficient virus exposure to susceptible neonatal calves. These studies would suggest that persistent BCV infections may play a role in the transmission of virus to new susceptible hosts.
The virus is widespread and antibody titers can be detected in the majority of adult cattle. Also, little difference has been detected, antigenically or biologically, between BCV of calves which is endemic on many farms and BCV isolated from the more sporadically occurring WD outbreaks of adult cattle. Therefore, it may be possible that WD is an expression of disease due to recrudescence of persistent BCV infection in adult cattle rather than an acute infection due to introduction of novel virus into the population. Demonstrating \textit{in vivo} persistent BCV infection, and understanding the viral and host cell factors involved, would greatly enhance our understanding of the epidemiology and pathogenesis of BCV infection, perhaps leading to new methods of BCV associated disease control. Further, the possibility of persistent BCV infection in adult cattle is important when considering the study design of future causal investigations of WD, since demonstration of infection in affected animals, especially with increasingly sensitive assays, may not be sufficient causal criteria.
2.8 References


CHAPTER 3

CONCEPTS OF CAUSATION AND THEIR APPLICATION TO WINTER DYSENTERY RESEARCH

While the objective of some studies are limited to describing observations without inference, the nature of most research in the biomedical fields is causal investigation. The objective of causal research is to seek the determinants of various effects, to gain understanding of causal relationships, and to estimate the magnitude of the effect produced by the causes.\(^1\) How to go about making causal inferences is by no means without controversy. Epidemiologists, microbiologists, statisticians, and philosophers continue to debate these issues, without consensus.\(^2\)

3.1 What is a cause and how do we measure it?

Holland tells us that Aristotle characterized 4 causes of a thing: the material cause as what the thing was made of, the formal cause as what the thing is made into, the efficient cause as that which makes the thing, and the final cause as that for which the thing was made. From Aristotle’s terminology, biomedical researchers are most interested in the efficient cause.\(^1\) In 1690, John Locke proposed a definition of cause as “that which produces any simple or complex idea,” and effect as “that which is
produced."¹ Some hold the notion that causes are active, and effects passive; so that a cause is not a characteristic, but more like a treatment that is potentially exposable to all of the units in the population.¹ Another view defines causes as active, and determinants as any factor that affects an outcome. Others choose to define all causes under the broader definition of determinants.³ Rothman defines a cause as "an act or event or a state of nature which initiates or permits, alone or in conjunction with other causes, a sequence of events resulting in an effect."⁴ Rothman describes a compelling model of causation incorporating the concept of sufficient cause:

A cause which inevitably produces the effect is sufficient...Common usage makes no distinction between that constellation of phenomena which constitutes a sufficient cause and the components of the constellation which are likewise referred to as "causes". Another qualification for sufficient causes is restriction to the minimum number of required component causes; this implies that the lack of any component cause renders the remaining component causes insufficient. Thus, measles virus is referred to as the cause of measles, whereas a sufficient cause for contracting measles involves lack of immunity to measles virus and possibly other factors in addition to exposure to measles virus. The term cause, then does not specify whether the reference is to a sufficient cause or to a component of a sufficient cause.

Most causes that are of interest in the health field are components of sufficient causes, but are not sufficient in themselves. Drinking contaminated water is not sufficient to produce cholera, and smoking is not sufficient to produce lung cancer, but both of these are components of sufficient causes. Identification of all the components of a given sufficient cause is unnecessary for prevention, in that blocking the causal role of but one component of a sufficient cause renders the joint action of the other components insufficient, and prevents the effect.⁴

Finally, the effect that a cause produces is measured relative to other causes. This line of thinking is easiest to follow in the context of an experiment, where we
measure the effect of a treatment (cause) in comparison to other treatments or non-treatment (different causes).\textsuperscript{1}

Rothmans model of sufficient cause is useful for understanding some of the measures of causation in biomedical research. The etiological fraction, or population attributable risk, of a component cause is a measure of the fraction of the disease resulting from the sufficient cause(s) to which the component cause belongs. This is a measure of the public health importance of the component cause,\textsuperscript{4} or how much disease could be prevented if the component cause could be prevented.\textsuperscript{5} Each component cause has its etiologic fraction and since the component causes can be part of more than one sufficient cause for a disease the sum of etiologic fractions of a set of component causes can be greater than 100 percent.\textsuperscript{4}

The risk of an effect, for an individual, depends on whether or not a sufficient cause has or will be formed; and so, for the individual is either 1 or 0. Risk for individuals is often expressed as the mean risk (the expected proportion of individuals that form sufficient causes) for a group of similar individuals. In this manner an individual’s risk is presented as a probability statement about the likelihood of forming a sufficient cause within a defined time period.\textsuperscript{4}

Since risk is presented as a probability statement it follows that, the presence of a risk factor, A, which requires other component causes of low prevalence to complete the sufficient cause will increase the probability of the effect (the risk) only slightly. Risk factor A then appears as a weak cause of the effect, because that sufficient cause
will only rarely be completed even though risk factor A may be observed more frequently. However the appearance of another risk factor, B, which requires other nearly ubiquitous component causes to complete the sufficient cause, will appear to be a strong cause because the sufficient cause will be complete nearly every time risk factor B is present. So the apparent strength of a causal risk factor depends on the prevalence of the complementary component causes in the same sufficient cause. Therefore, the strength of a causal risk factor, as measured by the risk ratio, is dependent on the relative distribution of the other causal factors within that population. The distribution of causal factors may vary from one group to another because of custom, circumstance or chance, therefore the strength of a causal risk factor is informative only within a given population with a given distribution of causal factors, but has no meaning in terms of the basic biology of the causal relationship.4

How inferences about causal relationships are made should be considered. It may be useful to briefly summarize the chronological development of some of the more influential concepts of causal inference in the fields of biology and philosophy. The purpose of this chronology is to form a basis of comparison, and to present the reader with some insight into the evolution of causal criterion as it relates to biomedical research; it is not a complete treatise on the subject.

3.2 The philosophy of causal inference

According to Rothman, the Greek philosophers followed the doctrine of rationalism, where scientific knowledge is accumulated through reason and intuition,
rather than observation. Later, in the 18th century, John Locke, Francis Bacon, and David Hume pioneered empirical reasoning. Empirical reasoning proposes to gain new knowledge by observation. Francis Bacon championed the scientific method, he felt that the method of science began with observations, from which generalizations can be derived (induction). However, Bacon's description of the scientific method has been faulted because good observations are rarely made without knowing what to look for, and while we can rationalize our observations, it is more difficult to come to rational certainty.

David Hume contributed much discussion to issues of causal inference. Hume recognized 3 criteria for causation: 1) cause and effect occur in a spatial/temporal relationship; 2) the cause must precede the effect; and 3) the cause and the effect occur together. Hume held that all knowledge comes from experience, but argued that, while we can observe that the proposed cause precedes the proposed event, the cause of an effect is not empirically verifiable. Even though we are tempted to make conclusions based on repeated observation, Hume argued that we never can know if our observations are coincidental or that we have simply failed to observe the exception. Hume's problem formed the basis for much of the future debate on the role of induction in causal inference.

In the 20th century, Popper discussed issues of causal inference, and rejected inductive inference as a myth. Popper addressed Hume's problem by claiming that the only way to advance knowledge was by conjecture (deduction) and then refutation.
Thus I was led by purely logical considerations to replace the psychological theory of induction by the following view. Without waiting, passively, for repetitions to impress or impose regularities upon us, we actively try to impose regularities upon the world. We try to discover similarities in it, and to interpret it in terms of laws invented by us. Without waiting for premises we jump to conclusions. These may have to be discarded later, should observation show that they are wrong.

This was a theory of trial and error -of conjectures and refutations. It made it possible to understand why our attempts to force interpretations upon the world were logically prior to the observation of similarities. Since there were logical reasons behind this procedure, I thought that it would apply in the field of science also; that scientific theories were not the digest of observations, but that they were inventions -conjectures boldly put forward for trial, to be eliminated if they clashed with observations; with observations which were rarely accidental but as a rule undertaken with the definite intention of testing a theory by obtaining, if possible, a decisive refutation.

Popper’s ideas have been both welcomed and viewed with skepticism by biomedical researchers. Some object to Popper’s philosophy because he suggests a conjecture is strengthened by failure to be refuted, yet fails to acknowledge this process as verification and induction, and also argues that falsification is not infallable because our observations are not perfect. Others note that most common and accepted epidemiological methods employ validation, and add that Popper’s process of conjecture and refutation is not, by itself, sufficient for causal inference, because it does not aid in timely decision making. Some have come to conclude that the acceptance or rejection of causal hypotheses comes about through a consensus of the scientific community which occasionally make dramatic shifts when new hypotheses cannot be rejected, but are inconsistent with older thinking.
John Platt simply called for research that, by thoughtful construction of hypotheses, and rigorous testing leads to strong inference.

...I will mention one severe but useful private test. -a touchstone of strong inference -that removes the necessity for third-person criticism, because it is a test that anyone can learn to carry with him for use as needed. It is our old friend the Baconian “exclusion,” but I call it “The Question.” Obviously it should be applied as much to one’s own thinking as to others’. It consists of asking in your own mind, on hearing any scientific explanation or theory put forward, “But sir, what experiment could disprove your hypothesis?”; or, on hearing a scientific experiment described, “But sir, what hypothesis does your experiment disprove?”

3.3 Biomedical science and causation

In some disciplines, such as mathematics, knowledge is self-contained. So that after an initial set of postulates are formed, all knowledge is derived from them. This process of deriving more specific knowledge from generalities is known as deduction. Biological knowledge, on the other hand is not inclusive, but is dependent upon observation. Making causal inference from repeated observations is termed induction. The inductive process of empirically gaining causal knowledge from observation is not a perfect process, because our observations are fallible. Rothman points out that the process of causal inference from observation is a device inherently used by humans (and perhaps animals) as a survival trait. In Hippocratic writings stagnant water in wet marshes and lakes was causally inferred to lead to diseases like malaria. These inferences, some true, many false, were based on observation, with little or no understanding of the underlying mechanisms of the disease. Biomedical knowledge progressed slowly, Vesalius published his observations on anatomy in 1543, and
Harvey, his observations of the circulation of the blood in 1628. Concepts of the function of the body, and disease were beginning to shape. In 1662, Gaunt used quantitative methods to make inferences from observations of death records in London. These quantitative methods were refined by Chadwick, Farr, and Snow, and used successfully to make causal inference about diseases, at a time when the miasma theory was predominant and the germ theory just gaining a foothold.*

In 1840, more than 4 decades before the first bacillus was ever isolated, Jakob Henle set requirements for making inferences that a particular agent was causally associated with a disease process. Henle suggested that if an agent is the cause of disease in one individual it should also be capable of causing disease in another individual.7,18,19 Later, as the work of Louis Pasteur was popularizing the germ theory of disease causation, Henle’s postulates were being applied by his former pupil, Robert Koch, to infer disease causation to several microorganisms. The Henle-Koch postulates became a classical point of reference in attributing cause of disease to infectious agents, even though Koch himself never regarded them as rigid criteria.18 Evans paraphrased the Henle-Koch postulates this way:

1) The parasite occurs in every case of the disease in question and under circumstances which can account for the pathological changes and clinical course of the disease.
2) It occurs in no other disease as a fortuitous and nonpathogenic parasite.
3) After being fully isolated from the body and repeatedly grown in pure culture, it can induce the disease anew.20

As the Henle-Koch postulates redefined concepts of disease causation, the way we defined diseases changed. For example, diseases, which had been described based on
the similarity of clinical signs, were redefined based on the causal organism. The
disease once known as phthisis, was reclassified into several diseases when the tubercle
bacillus was identified the causal agent of parts of the syndrome we recognize as
tuberculosis. 8

The work of Pasteur and Jenner introduced still newer concepts of disease
causation such as host immunity and with continuing discoveries of newer types of
agents and greater understanding of causal mechanisms the Henle-Koch postulates
were found to be limited in value as a means of validating causal hypotheses.18-20 Some
of the limitations of the Henle-Koch postulates were summarized by Evans: 1) the
postulates are not applicable to all pathogenic bacteria; 2) they may not apply to
viruses, fungi, or parasites; and 3) they do not allow for concepts such as: a)
asymptomatic carriers, b) a biological spectrum of disease, c) epidemiologic causation,
d) immunologic causation, e) that prevention by elimination of the cause is a
demonstration of causation, f) multiple causes, g) one recognized syndrome may have
different causes under different settings, h) latency of infection leading to disease, i)
immunopathology as a cause of disease.18

While fulfillment of the postulates is grounds for accepting a causal role, lack of
fulfillment may not exclude a causal relationship.18 Today the paradigm of the germ
theory has been replaced by the theory of multiple causation. To Koch's attribution of
the tubercle bacillus as the cause of tuberculosis, we now add such factors as family
exposure, genetic susceptibility, poor nutrition, and overcrowding.14
3.4 Methods of causal investigation

It appears that the final word on causal inference has not yet been written, yet in spite of this uncertainty, science progresses. While perhaps imperfect, some methodological guidelines for causal inference in the biomedical fields have been offered.

Susser acknowledges the fallibility of the inductive process, but comments:

In short, even if Popper's arguments were entirely sound (and several philosophers do not agree that they are), he offers an ideal model. This model is founded on logic and exemplified in the main from physics; it is not a working model founded on the realities of the epidemiologic enterprise. Of necessity scientists are pragmatists. When they must ignore philosophers they do, and they proceed with the business of verification and of induction as well as with more formal attempts at falsification by deduction.14

Controversy over the causal relationship of smoking and lung cancer has fueled much of the thinking of causal inference and validation of causal hypotheses. From this debate, Bradford Hill provided 9 criteria that he felt were useful, but neither necessary nor necessarily sufficient for validation of causal hypotheses. They are:

1) Strength of association, the cause and effect are observed together; 2) Consistency, that the association is regularly observed; 3) Specificity, that a cause has only one effect; 4) Temporality, that the cause precedes the effect; 5) Biological gradient, incremental changes in the cause lead to incremental changes in the effect; 6) Plausibility, that the proposed association is in accordance with our knowledge of biology; 7) Coherence, that the proposed association is in line with the findings of
others; 8) Experiment, that observations demonstrate the causal relationship; and 9) Analogy, that similar causal pathways have been demonstrated.21

Susser described 3 properties of a causal relationship, that are useful measures for validation of causal hypotheses: association, time-order, and direction.3 Association means that a causal factor must occur together with the effect. The association of a putative cause and effect is judged by probability of their occurrence together compared to prior expectations or chance and often determined by various statistical procedures. Association can also sometimes be inferred from experimental replication. Time-order means that in a causal association, the cause precedes (or is simultaneous with) the effect in time. If the reverse is demonstrated then causation is refuted. Time-order is assessed by appropriate study designs that allow observation of the relevant factors at the opportune times. Direction means that the effect is a consequence of the putative cause and hinges on the demonstration that a change in outcome (effect) is the result of a change in the cause. Direction is asymmetric in that changes in the cause lead to changes in the effect, but changes in the effect do not lead to changes in the cause.3

In the 1950's the question of the appropriateness of the methodologies used to address causal investigations was discussed by Hill,22 and then Cornfield23. Both eloquently commented on the value of both the experimental and observational methods, relating the successes, and lamenting the poor applications, of both approaches. Hill, a strong proponent of experimental methods comments that “...a poor
experiment serves no purpose. Yet it seems that the very magic in its name may serve
to mislead those who worship at the experimental shrine.” He then goes on to
comment about observational studies:

The observer may well have to be more patient than the experimenter - awaiting
the occurrence of the natural succession of events he desires to study; he may
well have to be more imaginative - sensing the correlations that lie below the
surface of his observations; and he may well have to be more logical and less
dogmatic - avoiding as the evil eye the fallacy of post hoc ergo propter hoc, the
mistaking of correlation for causation.\textsuperscript{22}

In contrasting the experimental and observational approaches, Cornfield writes:

Having thus argued that there is no difference in kind between the two types of
evidence it is of course necessary to add that there is a very important
difference in degree. It is a good deal more difficult to control variables in
observational than experimental material, so that the experimental method has
unraveled and will continue to unravel mysteries before which uncontrolled
observation would be powerless. But there is no difference in principle. There
are no such categories as first-class evidence and second-class evidence. There
are merely associations, whether observational or experimental that, in a given
state of knowledge, can be accounted for in only one way or in several different
ways. If the latter, it is our obligation to state so. To distinguish between
statistical association on the one hand and relationships that are established by
experimentation on the other, without any reference to alternative variables that
are present in one case but not the other, seems to us to be neither good
statistics, good science, nor good philosophy - though it may be good red
herring.\textsuperscript{23}

3.5 Conclusion

So we are left to conclude that both experimental and observational
investigation have made, and will continue to make, contributions to the advancement
of biomedical knowledge. Winter dysentery (WD) has been studied for over 65 years,
yet the cause(s) of this disease are unclear. The limitations of Koch’s postulates for
making causal inferences of WD have been discussed.\textsuperscript{24} Future causal investigations of
WD will require conjectures and careful construction of causal hypotheses, and then consideration of the appropriate study designs to test those hypotheses. Bovine coronavirus (BCV) has been conjectured to be causally associated with WD, yet no measure of association, time-order, or direction has been directly demonstrated. Other risk factors have been conjectured: host immunity, herd immunity, herd size, and housing factors. Other unidentified factors may yet be conjectured. Clearly there is much yet to do.

Field investigations have been and will certainly continue to be important methods of investigation for WD. Modern methods of field investigation in livestock enterprises have been reviewed. Outbreak investigations present special problems of investigation because of the associated time constraints, and case-control studies are especially well suited for such investigations.

Causal hypotheses about WD can be tested with careful thought and appropriate study design, both observational and experimental, using processes of refutation where possible, or validation if necessary. From these findings, new conjectures or modified hypotheses can be devised and tested; and through this continuing process our knowledge of the causal pathways of WD can be elucidated.
3.6 References


CHAPTER 4

EVALUATION OF TWO ANTIGEN CAPTURE ELISAS USING POLYCLONAL OR MONOCLONAL ANTIBODIES FOR THE DETECTION OF BOVINE CORONAVIRUS

4.1 Summary

Bovine coronavirus is an important cause of neonatal calf diarrhea. Investigators worldwide have suggested an association of bovine coronavirus with the syndrome of winter dysentery in adult dairy cattle. Detection of bovine coronavirus by veterinary diagnosticians and epidemiologists requires a rapid, reliable screening test, applicable to large numbers of samples. Two antigen capture ELISAs were developed and evaluated: the first utilizing polyclonal antibodies; and the second using a monoclonal antibody pool. Both assays detected bovine coronaviruses adapted to cell culture with similar sensitivity from dilutions of 2 strains from calf diarrhea and 7 strains from adult cattle during winter dysentery outbreaks. Fecal samples, known to contain bovine coronavirus by using electron microscopy or fluorescent antibody techniques (calf and winter dysentery bovine coronavirus strains), from 17 gnotobiotic and 43 field case calves were tested by both assays. The ELISA utilizing monoclonal antibodies had greater sensitivity and specificity than the other ELISA (97.2% and
100% versus 80.6% and 95.8%, respectively). Kappa values, comparing agreement of the assays to electron microscopy and immunoelectron microscopy results, were 0.96 for the ELISA using monoclonal antibodies and 0.75 for the ELISA using serum. Because of the excellent agreement with electron microscopy results, and the rapidity by which large numbers of samples may be diagnosed, the ELISA using monoclonal antibodies may be useful to diagnosticians and epidemiologists for routine detection of bovine coronavirus infections in neonatal calves and adult cattle.
4.2 Introduction

Bovine coronavirus (BCV) is recognized as a common causative agent of neonatal calf diarrhea. It has also been incriminated by several researchers from around the world as a causative agent for winter dysentery (WD), an acute diarrheal disease of adult cattle. Review articles concerning BCV and the immunology of coronaviruses have recently been published. Currently, the diagnosis of BCV infection is usually made by identifying the virus in feces. Because isolation of BCV in cell culture is difficult, this method is rarely employed as a diagnostic test; electron microscopy (EM) is often used for the identification of BCV in feces. Although the intact virion of BCV is fairly characteristic in appearance, it is not uncommon for the identifying surface projections of the virus to be lost during sample preparation or storage, making it more difficult to properly identify virus particles by EM.

Immunoelectron microscopy (IEM), utilizing specific antibodies against BCV, is used to increase the sensitivity and specificity of EM. Enzyme-linked immunosorbant assays (ELISAs) have been described for the detection of BCV antigen in feces; but, these assays have lacked sensitivity when compared to similar assays for other enteric pathogens. The use of monoclonal antibodies rather than polyclonal antibodies has increased the sensitivity and specificity of BCV ELISAs. ELISAs offer an advantage over EM and IEM of being able to rapidly evaluate large numbers of samples. This advantage is important for epidemiologists conducting large surveys and diagnosticians who must examine many submissions.
In this report we describe the development and evaluation of 2 ELISAs for detection of BCV antigen in feces. In 1, polyclonal antibodies were used for antigen capture (PACELISA); the other was identical except for the use of monoclonal antibodies (MAbs)(MACELISA). We also present new information related to evaluation of the two assays for detection of both calf and WD BCV strains and the sensitivity and specificity obtained with the MACELISA.

4.3 Materials and Methods

Nine strains of BCV adapted to cell culture in human rectal tumor (HRT-18) cells, as previously described were used to evaluate the ELISAs. Of the 9 strains evaluated, 2 (Mebus, DB2) were isolated from diarrheic calves and 7 (DBA, SD, BE, BM, AW, TS, CN) were isolated from adult cows clinically affected with WD.

Sixty reference fecal samples were collected from gnotobiotic calves or field cases of neonatal calf scour for which the BCV infection status was determined by EM, IEM, or immunofluorescence. These samples were diluted 1:25 in phosphate buffered saline (PBS), and centrifuged (850 x g, 20 minutes), and the supernatant was saved for ELISA testing. Of these 60 fecal samples, 36 BCV positive samples were obtained from 16 gnotobiotic calves experimentally infected with BCV and from 20 field cases of calf diarrhea. All 20 of the BCV positive field samples were concurrently infected with rotavirus as determined by EM. Twenty-four BCV negative fecal samples were obtained from 23 field cases of calf diarrhea and 1 gnotobiotic calf. All 23 BCV negative field samples were from diarrheic calves infected with one or more
neonatal diarrheal pathogens (rotavirus, n= 16; Bredavirus, n=1; cryptosporidia n=7).

In addition to the 60 fecal samples described, 2 Bredavirus positive fecal samples (by IEM) from gnotobiotic calves infected with Bredavirus were tested to determine the ability of the MACELISA to distinguish this morphologically similar but antigenically distinct virus from BCV. Fecal samples and cell culture passaged virus aliquots were stored at -70°C until prepared for testing.

Hyperimmune serum prepared in a gnotobiotic calf in response to the Mebus strain of BCV was used for the polyclonal antibody positive coating. This serum had a virus neutralization (VN) antibody titer to Mebus BCV of 1:32,000. For negative coating, serum from a newborn gnotobiotic calf free of BCV antibodies was used. Both sera were diluted 1:2 with glycerol and stored at -20°C.

MAbs against the virulent DB2 strain of BCV were prepared in mice as previously described. Antibody titers of the MAbs to the Mebus strain of BCV were determined by VN and indirect immunofluorescence assays. The BCV protein specificity of the MAbs was determined by Western Blot assay against the Mebus strain of BCV (Heckert and Saif, unpublished). Ten MAbs with the highest antibody titers to BCV, as determined by indirect cell culture immunofluorescence assay were chosen for further evaluation as positive coating for ELISA. The MAbs were evaluated as diluted ascitic fluid in the ELISA, first individually against cell culture adapted strains of BCV and then as pools of 3, with each pool evaluated containing MAbs directed against 3 BCV structural proteins (nucleocapsid, N; spike protein, S; hemagglutinin
esterase, HE). Titers to DBA, SD, and Mebus strains of BCV, determined by indirect immunofluorescent antibody tests, were 1:25,600, 1:51,200, and 1:25,600, respectively for the MAb directed against the HE viral protein; 1:51,200 for all three viral strains for the MAb directed against the S viral protein; and ≥1:102,400 for all three strains for the MAb directed against the N viral protein. Negative coating was diluted ascitic fluid from mice inoculated with SP2/0 mouse myeloma cells.

Immunoelectron microscopy was conducted using procedures similar to those described previously. Fecal samples were diluted 1:5 in PBS, sonicated and clarified at 4°C and the supernatants filtered through 0.45 μm syringe filters. Diluted gnotobiotic calf anti-BCV serum was added to the supernatants and incubated overnight at 4°C. The immune complexes were pelleted by centrifugation (69,000 x g, 35 minutes, 4°C.) and then resuspended in sterile distilled H₂O (dH₂O), repelleted as above, suspended in 50 μl dH₂O and vortexed. For EM, one drop of 3% PTA, pH 7.0 was added and one drop of the suspension placed on formvar-coated carbonized copper grids.

The ELISAs described were indirect, double antibody sandwich, antigen-capture assays. In the PACELISA paired rows of a 96 well microtiter plate were coated with 100 μl /well of a 1:4000 dilution of polyclonal gnotobiotic calf hyperimmune anti-BCV serum (B6429, positive coating), or a 1:4000 dilution of BCV antibody negative serum (negative coating), in carbonate-bicarbonate buffer. Plates were incubated at 4°C overnight, or stored at 4°C for up to 7 days. After washing,
200 μl of 5% non-fat dry milk in PBS was added to each well as a blocking step to minimize non-specific binding and the plates were incubated for 2 hours at 25°C. The plates were washed and 100 μl of the test samples (cell culture virus or 1:25 dilutions of feces) were placed in paired wells of BCV antibody-positive and -negative serum coating. Plates were incubated at 4°C for 16 hours. The plates were washed and 100 μl of guinea pig anti-BCV (Mebus) serum diluted 1:4000 in PBS/0.05% Tween 20/2% bovine serum albumin was added to each well. The plates were incubated 1 hour at 25°C and washed, and 100 μl of a 1:4000 dilution of sheep anti-guinea pig peroxidase-conjugated antibody in PBS/2% bovine serum albumin was added to each well. Plates were incubated 1 hour at 25°C, and washed. One hundred microliters of the chromogen substrate, a 1:1000 solution of H₂O₂ and 2,2'-azino-bis(3-ethyl-benzthiazoline)sulfonic acid in 0.1M sodium citrate was applied to each well. After 20 minutes, the color reaction was stopped by the addition of 50 μl of 5% sodium dodecyl sulfate per well. The absorbance value of each well was read at a wavelength of 414 nm. with a computer linked ELISA plate reader and the readings were saved as ASCII files.

At each washing, the plates were rinsed 5 times with PBS/0.05% Tween 20. Plates were sealed with ELISA plate tape during each incubation step. A fecal sample from a gnotobiotic calf infected with BCV and determined positive for virus by IEM was used on each plate as a positive control. The negative control fecal sample was from a diarrheic field calf determined negative for BCV by IEM.
The MACELISA procedure was identical to the PACELISA except MAbs to BCV were substituted for the polyclonal antibodies for coating the plates. In the MACELISA a pool of 3 MAbs directed against 3 structural BCV proteins (N, S, HE) was used as positive coating. One hundred microliters of a 1:8000 dilution of the 3 MAbs, used as mouse ascites fluids, was added to paired rows as positive coating. Similarly, 100μl of a 1:8000 dilution of BCV antibody negative mouse ascites fluids was added to paired rows as negative coating.

A spreadsheet program was used to calculate the ELISA value for each sample. The mathematical calculation of the ELISA value for each fecal sample was the average absorbance of the paired positive antibody coated wells minus the average absorbance of the paired negative antibody coated wells.

To determine the analytical sensitivity of each ELISA to detect BCV antigens, serial dilutions of 9 cell-culture adapted strains of BCV were tested in both assays. The TCID$_{50}$ of BCV in cell culture medium was determined by the Reed-Münch method, and 100 μl of 4-fold serial dilutions of the cell culture BCV suspensions in PBS were applied to antibody-positive coated wells. The ELISA value used for detecting the cell culture BCV titer endpoint, was defined as the absorbance value of virus diluted in PBS on positive coating minus the absorbance of PBS with no virus. The virus dilution at which the ELISA value was greater than an empirical 0.007 was determined to be the endpoint and expressed as the minimum TCID$_{50}$ detectable.
For each assay, the frequency distribution of the ELISA values for 60 BCV-positive or -negative fecal samples, previously described, were calculated and graphed. From the frequency distribution data, the sensitivity and specificity at each ELISA value point was calculated and graphed. The mathematical product of sensitivity times specificity, termed efficiency, was calculated and graphed for each ELISA value to provide the probability of correct classification given a single sample of unknown status (Hancock, 1994, personal communication). Each ELISA cut-off value was optimized at the point of greatest efficiency. Using the cut-off value determined for each assay, the optimum combination of sensitivity and specificity of each assay was determined.

Fifty six (33 BCV-positive samples and 23 BCV-negative samples) of the 60 reference fecal samples were tested using EM or IEM. Using the same previously determined ELISA cut-off values, and the results from these 56 fecal samples, a Kappa value was calculated for both the PACELISA and the MACELISA to measure the agreement of each ELISA with EM/IEM results.

4.4 Results

Both assays detected all 9 BCV strains from cell culture. Both assays detected each strain of cell culture virus at similar endpoints of virus titer (Fig. 4.1). There was up to a \(2\ \log_{10}\) difference in the analytical sensitivities among various strains with generally greater analytical sensitivity of both assays with the calf strains of BCV (DB-2, Mebus) than with the WD strains. The PACELISA had analytical sensitivities
expressed in minimum TCID<sub>50</sub> detectable, for each strain as follows: DBA, 10<sup>5.1</sup>; SD, 10<sup>5.8</sup>; CN, 10<sup>4.9</sup>; BE, 10<sup>4.4</sup>; BM, 10<sup>5.5</sup>; AW, 10<sup>5.5</sup>; TS, 10<sup>3.9</sup>; Mebus, 10<sup>3.8</sup>; and DB-2, 10<sup>4.4</sup>. Similarly, the analytical sensitivities for MACELISA were: DBA, 10<sup>4.5</sup>; SD, 10<sup>4.8</sup>; CN, 10<sup>4.9</sup>; BE, 10<sup>4.4</sup>; BM, 10<sup>4.9</sup>; AW, 10<sup>4.9</sup>; TS, 10<sup>3.9</sup>; Mebus, 10<sup>3.8</sup>; and DB-2, 10<sup>3.8</sup>.

The frequency distributions of the values from MACELISA and PACELISA, obtained from the 36 BCV-positive and 24 BCV-negative reference fecal samples, are illustrated in Fig. 4.2. There was greater differentiation in the frequency distribution of values from positive and negative samples with the MACELISA than with the PACELISA (Fig. 4.2). Utilizing the calculations for efficiency, the optimum cut-off value for the PACELISA was 0.110 (Fig. 4.3A); the optimum cut-off ELISA value for the MACELISA was 0.030 (Fig. 4.3B). At these cut-off values for the calf fecal samples, the sensitivity of the PACELISA was 80.6% (95% confidence interval of 91.2% - 63.4%) and the specificity was 95.8% (99.8% - 76.9%); for the MACELISA the sensitivity was 97.2% (99.9% - 83.8%) and the specificity was 100% (100% - 88.3%). The 2 additional Bredavirus IEM positive fecal samples from gnotobiotic calves were negative for BCV antigens when tested by MACELISA (values of 0.013 and 0.022).

Kappa values measuring agreement with EM/IEM results at the optimum cut-off value for MACELISA and PACELISA were 0.96 (95% confidence interval of 1.0 to 0.89,) and 0.75 (0.92 to 0.58), respectively, for the calf fecal samples.
4.5 Discussion

Subtracting the background value, the nonspecific reaction occurring within the plates, from the signal, left the ELISA value which represents the specific antigen-antibody reaction occurring within the wells. To determine the comparable end point of virus detection by both ELISAs, cell culture BCV was serially diluted with PBS. The background value in this case was considered to be the absorbance value of PBS on positive coating. By subtracting the absorbance value of PBS on the positive coating to calculate the ELISA value, we were able to compare the effectiveness of the two positive coatings for attracting antigen; positive ELISA values indicate the detection of BCV antigen as compared to PBS with no virus.

Fecal samples present a more difficult challenge for the correct interpretation of ELISA results. Feces contain biologic and immunologic constituents that may effect the occurrence of non-specific reactions, so that each fecal sample will have a unique background absorbance value associated with it. To minimize the effect of background, BCV antibody-negative coating was utilized with the assumption that the absorbance value obtained from the negative coating represents the non-specific reactions that also take place on the positive coating. The ELISA value calculation we used for fecal samples essentially blanks each fecal sample for its own unique background.

It is not uncommon for biologic data to have non-gaussian distributions. The method of determining the optimum cut-off value employed uses a non-gaussian
method that relies on the frequency distribution of known BCV-positive and -negative fecal samples to determine the sensitivity and specificity of the assays at each ELISA value interval. At the point of maximum efficiency the fewest errors, both false positive and false negative, are made. This point intuitively presents itself as the optimum point for the cut-off value assuming that the cost of either error is the same.

Both assays demonstrated excellent specificity. All of the BCV-positive and -negative reference samples from field cases were from diarrheic calves with other concurrent enteric infections. It is reasonable to expect that diagnostic fecal samples from calves with neonatal diarrhea also will have more than one pathogen present.\textsuperscript{31,32} Both tests exhibited the ability to identify BCV antigen in feces to the exclusion of other calf enteric pathogens. Bredavirus is morphologically similar, but antigenically distinct from BCV, and has been incriminated in WD outbreaks;\textsuperscript{4} therefore, we used the MACELISA to test 2 fecal samples from gnotobiotic calves that contained Bredavirus and were negative for BCV by IEM. These two samples were negative by MACELISA, indicating that the presence of Bredavirus in field specimens did not give false-positive results.

The sensitivity and specificity of the MACELISA were not statistically different from those of the PACELISA. However, upon examination of the distribution of the values obtained by both assays using the same samples, the MACELISA appeared to more clearly differentiate BCV-positive samples from BCV-negative samples (Fig. 4.2). This clear distinction of negative and positive samples with the use of MAbs rather than
polyclonal antibodies is probably a result of the advantages of specificity of binding and homogeneity. Thus, the problems of cross-reactivity of naturally occurring polyclonal antibodies are minimized. Other advantages associated with the use of MAbs over polyclonal antibodies are that uniform reagents are consistently available, leading to reproducible results from assay to assay and laboratory to laboratory. The use of a MAb pool directed at different epitopes gives the theoretical advantage of increasing the affinity for the antigen over single MAbs which can bind at only 1 site.

Evaluation of an assay by the calculation of sensitivity and specificity makes the assumption that the reference samples have been diagnosed without error by an infallible reference assay; rarely can this assumption be met. An alternative method of evaluating a new diagnostic assay is by calculation of the Kappa value. The kappa value is a measure of the diagnostic agreement between 2 tests beyond the agreement due solely to chance. Kappa values range from 1 to -1; a kappa value of 1 is interpreted as perfect diagnostic agreement between the two tests, -1 means complete disagreement, and 0 means no agreement between the two tests except that due to chance. The determination of the kappa value is an appropriate method for comparing a new diagnostic method (MACELISA and PACELISA) with traditional diagnostic methods (EM/IEM). A Kappa value of 0.96 for the MACELISA means excellent agreement with the results from EM/IEM procedures and demonstrates that MACELISA results can be equally reliable for the detection of BCV antigen in feces. The ability of MACELISA to accurately and reliably detect BCV antigen from WD
BCV strains in cell culture and from neonatal calf scours cases suggests that the assay will be useful to epidemiologists and diagnosticians investigating WD and calf BCV infections.

4.6 Acknowledgments

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4.7 Sources and Manufacturers

a. Uniflo®, Schleicher & Schuell, Keene, NH


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


e. AUTOmate v. 2.2; Flow Laboratories Inc., McLean, Va.

f Quattro-Pro windows v.5.0; Borland International Inc. Scotts Valley, Ca.
4.8 References


Figure 4.1 End point of virus titer detection by ELISA from 4 BCV strains in cell culture. The ELISA value for this comparison was calculated by subtracting the absorbance of PBS on positive coating from the absorbance of the cell culture virus diluted in PBS on positive coating. The positive coating was either a MAb pool (MAb pool 1) or gnotobiotic calf hyperimmune serum against BCV (B6429). An ELISA value of 0 is the value of the diluent (PBS) on positive coating: ELISA values >0.007 were considered positive.
Figure 4.2 Frequency distribution of ELISA values obtained by BCV antigen-capture ELISA from 36 BCV-positive and 24 BCV-negative reference fecal samples. A. ELISA with polyclonal antibodies (PACELISA). B. ELISA with MAbs (MACELISA).
Figure 4.3 Sensitivity, specificity, and efficiency of BCV antigen-capture ELISAs illustrating calculation of the optimum cutoff values (vertical lines) to minimize errors (false positive and false negative). A. ELISA with polyclonal antibodies (PACELISA). B. ELISA with MAbs (MACELISA).
CHAPTER 5

EPIDEMIOLOGY OF WINTER DYSENTERY IN DAIRY CATTLE: ASSESSMENT OF HERD-LEVEL CAUSATIVE AGENTS AND RISK FACTORS

5.1 Summary

Objective To test the strength of association between herd-level exposure to bovine coronavirus and winter dysentery and to explore other potential risk factors for winter dysentery in dairy herds.

Sample population Ohio dairy herds affected with winter dysentery served as cases. For each case, 2 unaffected herds from the same local area were used as controls.

Procedure Case-control study using herds as the unit of investigation. Multivariable logistic regression modelling was used to identify explanatory risk factors for disease.

Results Four herd-level exposures appeared to increase a herds risk for winter dysentery: 1) increasing herd prevalence of adult cows demonstrating a 4-fold or greater rise in bovine coronavirus IgG serum antibody ELISA titer; 2) increasing herd prevalence of adult cows demonstrating a 4-fold or greater bovine viral diarrhea virus serum neutralization titer; 3) housing cattle in tie stalls or stanchion barns rather than freestalls; and 4) using manure handling equipment to handle feed. The adjusted
population attributable risk for these variables was 71 %, 43 %, 53 %, and 31 % respectively and 99 % overall, suggesting that these variables had considerable impact on the winter dysentery experience of the study population.

**Conclusions and clinical relevance** In Ohio dairies, recent herd exposure to bovine coronavirus appeared to increase the risk for winter dysentery outbreaks. Also, some winter dysentery outbreaks might have been associated with acute bovine viral diarrhea infection; and certain housing and management practices may have increased the risk of winter dysentery.
5.2 Introduction

Winter dysentery (WD) is a disease of dairy and beef cattle recognized by farmers and veterinarians in many parts of the world. The disease is characterized by a sudden onset of diarrhea that rapidly affects many adult animals in the herd. Affected animals rarely die, but losses can be incurred from lost body condition and dramatic decreases in milk production. The disease syndrome is diagnosed by considering the clinical signs of affected animals and the herd outbreak history. Other diseases can appear similar to WD, including acute bovine virus diarrhea virus (BVDV) infections and salmonellosis. It is not clear what role infection with these agents may play in WD. Previous descriptive studies have suggested that the disease is infectious in nature; and, because of the similarities in clinical signs between outbreaks, that a single etiologic agent may be involved.

Etiologic investigations of WD have been reported since 1931. Since then, many researchers have investigated the role of various infectious agents in the disease; however, the causative agent of WD has not yet been definitively demonstrated. If the important risk factors for the development of WD could be determined, control measures might be adopted to prevent outbreaks of the disease. Such control methods could include the enhancement of immunity to specific agents through vaccination, or management modifications to minimize the risk of disease occurrence. Researchers from many parts of the world have found bovine coronavirus (BCV) in the feces of affected animals, and have demonstrated BCV antibody seroresponses during
outbreaks of WD. Diarrhea has been experimentally induced, though inconsistently, in adult cows after inoculation with filtered, and unfiltered feces from WD affected animals. Recently, diarrhea was experimentally induced in, and BCV antigen demonstrated in the feces of, 4 BCV seronegative adult animals following exposure to an experimentally BCV infected calf. However in spite of what appears to be considerable circumstantial evidence of a causal association between BCV and WD, only 1 controlled field investigation has been conducted specifically testing the role of BCV infection in WD outbreaks. That study, conducted in the Netherlands, found an association with a BCV-related virus, Bredavirus, and WD but did not demonstrate a statistically significant measure of association for BCV.

Controlled field studies of WD have suggested several non-etiologic risk factors for WD in herds; however, these findings have not been consistent and have sometimes been conflicting. In a prospective study of dairies in central Sweden, researchers found a herd-level cumulative incidence of 28.5 % for WD, with most outbreaks occurring between November and January, and increased severity of disease in herds not experiencing WD within the previous 4 years. In a survey study of farmer diagnosed WD in New York, researchers reported increased risk of WD with increasing herd size, and in herds experiencing outbreaks in previous years. Researchers in France reported greater risk in small herds with either greater than, or lesser than recommended stocking density.
This current investigation was undertaken to test the hypothesis that herd-level exposure to BCV is an important risk factor for WD outbreaks, and to generate hypotheses about other potential herd-level etiologic and non-etiologic risk factors.

5.3 Materials and Methods

Definition of disease

For the purpose of this study, case herds were defined as follows:

• The primary clinical sign was diarrhea in adult cattle.
• More than 15%, or a total of 15 animals, of the adult herd affected.
• Rapid spread of the disease within the herd. The majority of animals must have become ill within one week of the onset of clinical signs.
• The disease outbreak had less than 2% death loss.

In addition at least one of the following conditions must have existed:

• The time period of the outbreak must have been from November 1 to April 30.
• Blood present in the feces of some animals.
• Concurrent respiratory signs.
• The herd’s milk production decreased by at least 10%.

Study design

This case-control study was conducted during the period November 1992 to April 1994. Herds meeting the case definition were compared to control herds from the same geographical area, which had not exhibited adult-cow diarrhea that season. Data were collected from two control herds for every case herd. In case herd, approximately
10 affected and 10 non-affected animals were systematically-randomly selected for diagnostic sampling using the last digit of the animals identification number. Often, all of the affected animals were sampled, and if non-affected individuals were subsequently identified as becoming ill then they were reclassified as affected. In control herds, 10 animals were chosen for diagnostic sampling using the same systematic-random selection process used in case herds. In control herds, specimens collected during the first sampling were referred to as acute specimens, and later specimens were designated as convalescent.

A second blood sample was collected from all herds approximately three weeks after the initial visit. From the time of collection on the farm, feces and blood were maintained on ice, or refrigeration at 4°C. Within 24 hours of collection, the fecal samples were aliquoted and frozen at -70°C for later viral studies, or shipped overnight on ice for bacterial culture. Serum was separated, aliquoted, heat inactivated (56°C for 30 minutes) and then frozen. Whole blood samples were submitted immediately after arrival from the farm for BVDV detection during the first year of investigation.26 For BVDV detection in the second year, 100 ml of bulk tank milk was collected for polymerase chain reaction (PCR) testing27 in lieu of whole blood. Production and health records were collected during the initial visit to case and control herds and a questionnaire on herd management practices was completed by the investigator during the interview of the producer. A single investigator, (DRS), visited all farms, collected the diagnostic samples, conducted the interviews and made any subjective evaluations.
Case-herd selection

To increase awareness of the study, letters explaining our interest in investigating WD outbreaks were sent to Ohio veterinarians involved in dairy practice, and Ohio Cooperative Extension county agents, and the study was described in Dairy Herd Improvement Association (DHIA) and extension newsletters distributed to dairy farmers and veterinarians throughout the state. For inclusion in the study, case herds must have met the case definition, and the outbreak must have been currently ongoing. In case herds, the farmer was asked to provide lists of affected (diarrheic) and non-affected animals so that we could concurrently investigate cow-level risk factors for disease within WD affected herds.

Control-herd selection

Within 2-3 weeks after a case herd was identified, 2 control herds were randomly selected from a frame of dairies in the same area using a random number generator. If the case herd was on DHIA test then the frame for control selection was the list of DHIA dairies of the same breed in the same area. Two case herds were not DHIA members and their controls were selected from a frame of non-DHIA dairies in the same county that was provided by area veterinarians. Control herds must not have had any herd problem with diarrhea in adult cows during that winter season.

Diagnostic assays

Bovine coronavirus exposure was determined for each individual using 2 diagnostic assays. The first method, a double sandwich antigen capture ELISA,
detected the presence of BCV antigens in the feces. An ELISA value was calculated by subtracting the average optical density (OD) of the 2 negative coated wells from the average OD value of the 2 positive coated wells for each sample. An ELISA value greater than 0.100 was considered positive for BCV antigens.

The second BCV diagnostic assay was an ELISA to measure the serum BCV IgG antibody response from paired serum samples. Ninety-six well ELISA plates were coated with 100 μl of a 1:8000 dilution of 3 MAbs specific for BCV in carbonate-bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. Plates were washed 5 times between each step with 300 μl of a solution of phosphate-buffered saline (pH 7.4) and 0.05% Tween 20 (PBS/Tw). Next, 100 μl of a 1:2000 dilution of semi-purified WD-BCV virus in a solution of PBS/Tw was added to each well and incubated for 1h, 25°C. After washing, 100 μl per well of serial 2-fold dilutions (range of dilutions 1:400 to 1:6400) of the serum samples with PBS/Tw were applied in rows. Samples from individuals with out-of-range titers, were retested at dilutions of 1:50 to 1:51,200. One well at the end of each row was filled with 100 μl of PBS/Tw as a negative control. Plates with the sample dilutions were incubated for 1 h, 25°C and washed. One-hundred μl of a 1:128,000 dilution of rabbit anti-bovine IgG horseradish-peroxidase-conjugated antibody in PBS/Tw was added to each well, incubated 1 h at 25°C, then washed. One-hundred μl of the chromagen substrate, a 1:1000 dilution of 30% H₂O₂ and 2,2' azino-bis(3-ethyl-benzthiazoline) sulfonic acid in 0.1 M sodium citrate was applied to each well. The color reaction was stopped at 20 minutes with
the addition of 50 μl of 5% sodium dodecyl sulfate per well. The OD of each well was read at a wavelength of 414 nm. Acute and convalescent serum samples were run concurrently on the same plates. The endpoint of detection for each pair of samples was defined as 1.5 times the average OD of the negative control wells in the acute and convalescent rows for that animal. The titer for a sample was defined as the inverse of the interpolated dilution at the last point where the OD value dropped below the endpoint OD. The endpoint dilution was interpolated from the OD values at the dilutions above and below the endpoint OD and the logarithmic transformation of those dilutions, using the same principals that are used to calculate geometric mean titers. If the BCV antibody titer was below the level of detection; the titers were reported as the inverse of one-half of the lowest sample dilution tested. A BCV seroresponse was defined for each individual as a ≥4-fold (2 dilutions) rise in the convalescent BCV IgG antibody titer over the acute titer.

Herd exposure to BVDV was measured by several methods: 1) serum neutralizing antibody (SN) responses in paired sera; 2) virus isolation conducted on whole blood buffy coat cells from each individual in the first 27 herds; and 3) PCR testing of a bulk tank milk sample, collected at the initial farm visit, from the final 9 herds. The serum neutralization test was conducted by the microtitration method using varying serum dilutions (starting at 1:4) and 100 to 500 TCID₅₀ of the Singer strain of BVDV. Bovine turbinate cells free of BVDV were used to conduct the test.
A BVDV seroresponse was defined as a ≥4-fold increase in the convalescent BVDV SN titer over the acute titer.

Herd exposure to *Salmonella* spp. was determined by selective bacterial culture of the feces of each individual. Approximately 1 g of feces from each sample was placed into each of 2 culture media: tetrathionate broth (Tet) or GN Hajna broth (GN). All cultures were incubated overnight at 37°C. At 24 h, approximately 100 μl from the GN culture was transferred into Rappaport R-10 medium (R-10)(GN-R). At 48 h, 100μl was also transferred from the Tet culture into R-10 (T48-R). All GN-R and T48-R media were incubated overnight at 37°C, then streaked onto brilliant green agar with sulfadiazine (BGS) plates. Additionally, the 48 h Tet culture (T48) was also streaked onto BGS. All BGS plates were incubated overnight at 37°C. Colonies having the typical appearance of *Salmonella* were transferred to triple sugar iron and lysine iron agar slants. All slants were incubated overnight at 37°C. Presumptive positive isolates were serogrouped using serogroup specific typing sera, then sent to the NVSL for serotyping.

Heat-fixed fecal smears were acid-fast stained and examined at 400X by light microscopy for *Cryptosporidia* oocysts.

*Statistical analysis*

Each herd’s exposure to each infectious agent was reported as a proportion of the sample that tested positive, or as a geometrical mean titer for serological results. The prevalences and geometric mean titers from case herds were calculated in case
herds from the test information from the sick and the unaffected individuals. Titers
were logarithm transformed prior to statistical testing.

Univariable odds ratios (OR) were calculated for each variable using logistic
regression and conditional logistic regression, which considers matching in the study
design. Conditional logistic regression OR’s and their associated standard errors for
those variables representing herd exposure to infectious agents were compared to the
same statistics obtained by univariable logistic regression. Adjusted OR’s were
estimated by multivariable logistic regression using manual forward and backward
selection considering only the variables with -2 Log likelihood chi-square p-values less
than 0.25 as univariables. The main effect variables (related to BCV exposure) were
tested in the models at a -2 log likelihood $X^2$ p value of 0.05. Other variables were
allowed to remain in the models as hypothesis generating variables to a -2 log
likelihood $X^2$ p value of 0.10. In this fashion, the best 1, 2, 3, and 4 variable logistic
models were determined. The population attributable risk for the main effect and each
explanatory variable, and the summary attributable risk for the variables considered
collectively, was calculated using the results of the multivariable analysis as previously
described.30

5.4 Results

Twelve case herds and 24 control herds were studied from 9 Ohio counties. All
of the WD outbreaks occurred during the winter months. The median herd size was 57
adult animals for case herds (range 25 to 225) and 62 adult animals for control herds
One herd, randomly selected as a control, developed signs meeting our definition of WD 4 weeks later, and was again included in the study as a case herd. Most herds were comprised of Holstein cattle, but approximately one-half of the cows in one case herd were Jersey, and another case herd had several breeds represented. One control herd was comprised of Jersey cattle. The attack rate in case herds ranged from 15 % to 100 %. One case and 2 control herds regularly vaccinated dry cows against BCV to provide passive protection to neonatal calves (Table 5.1). All but 5 herds (all controls) vaccinated the adult herd at least annually using commercially available vaccines containing BVDV antigens. Diagnostic samples were collected from 469 animals representing 2559 adult animals from the 36 herds (Table 5.1).

The median period that clinical signs were observed in a herd was 6 days (range 3-10). The median time to the initial visit to case herds was 3 days (range 1-9) from the onset of the outbreak as reported by the owner. Convalescent samples were collected from case and control herds at a median of 24 days (range 18-37) after the initial visit.

The median number of cattle sampled was 20 in case herds (range 12 to 26). Ten animals were sampled in each of the control herds. The descriptive statistics for the BCV, BVDV and Salmonella diagnostic assays are summarized by herd (Table 5.1). Most animals in case and control herds had measurable acute antibody titers to BCV at the onset of the study. The median geometric mean BCV IgG antibody titer value was 1500 (range 370 to 3800) among case herds, and 2161 (range 849 to 9805)
among control herds. The median prevalence of BCV seroresponses was 5.8% (range 0% to 84.2%) within case herds and 0% (range 0% to 10%) within control herds. At least 1 individual had a measurable BCV seroresponse in 9 out of the 12 (75%) case herds and 2 out of the 24 (8.3%) control herds. The frequency distributions for BCV seroresponse prevalences illustrate the differences in BCV seroresponse between the 2 groups (Figure 5.1). At least 1 individual had ELISA positive evidence that BCV antigen was in the feces in 3 out of 12 (25%) case herds and 3 out of 24 (12.5%) control herds.

The median geometric mean BVDV SN titer value was 13.7 (range 3.7 to 66.3) among case herds, and 11.7 (range 2.0 to 52.0) among control herds. At least 1 individual had a measurable BVDV SN seroresponse in 7 out of 12 (58.3%) case herds and 10 out of 24 (41.7%) control herds. The median prevalence of BVDV seroresponse was 4.7% (range 0 to 33.3%) within case herds, and 0% (range 0 to 20%) within control herds. Bovine viral diarrhea virus was not isolated from the buffy coat cells of any individuals in any of the 27 herds so tested. Only 1 herd (a case herd) of the 9 was BVDV positive by PCR of bulk tank milk; no BVDV seroresponses were detected in that herd.

_Cryptosporidia_ organisms were not found in any of the 469 fecal smears.

_Salmonella_ was cultured from a total of 24 individuals in 2 case herds and 2 control herds. Interestingly, _Salmonella_ was cultured in the 1 herd that served as both a control and a case. In that herd _Salmonella_ was cultured from 7 (6 _S. cerro_, 1 _S.
montevideo) of 10 animals (70% total) when sampled as a control, and 9 (8 S. cerro, 1 S. montevideo) of 12 animals (75%) 4 weeks later when the herd experienced WD. In the other 2 herds with Salmonella culture positive animals 1 individual out of 23 (4.35%) in a case herd was cultured positive for serotype S. montevideo, and 7 individuals out of 10 (70%) in a control herd were cultured positive for serotype S. derby.

The unadjusted logistic regression OR's, with 95% confidence intervals for the variables measured are summarized (Figure 5.2). The unadjusted OR for each 10% increase in prevalence of the herd that demonstrated a BCV IgG antibody seroresponse was 15.5 (p<0.0001). Each log2 increase in a herd’s mean acute BCV IgG antibody titer had an unadjusted OR of 0.488 (p=0.039). The unadjusted OR for each 10% increase in prevalence of the herd that demonstrated a BVDV SN antibody seroresponse was 1.93 (p=0.136). The stocking density expressed as the number of adult cows per 100 square meters had an unadjusted OR of 5.6 (p=0.043). Herds that used manure handling equipment to handle feed had an unadjusted OR of 5.5 (p=0.0654). By conditional logistic regression, the unadjusted OR for each 10% increase in the herd prevalence of animals with a BCV IgG antibody seroresponse was 13.0 (p=0.0006). The unadjusted conditional logistic regression OR for each 10% increase in herd prevalence of animals with a BVDV SN antibody seroresponse was 1.97 (p=0.130).

The best 1-4 variable logistic models are summarized (Table 5.2). The variables that added significance to the best 4-variable model, adjusting for the effects...
of other important variables, were: 1) each 10% increase in a herds’ BCV seroresponse prevalence; 2) each 10% increase in a herds’ BVDV seroresponse prevalence; 3) if the herd was housed in tie-stall or stanchion barns, or tie-stalls and freestalls, compared to freestall housing alone; and 4) if the herd used manure handling equipment to handle feed.

The population attributable risk (etiologic fraction) for BCV exposure, as determined by seroresponse to BCV IgG antibody, was 71%. The population attributable risk for BVDV exposure, as determined by BVDV SN antibody seroresponse was 43%. Population attributable risks for use of tie-stalls or stanchion barns, and use of manure handling equipment to handle feed was 53% and 31% respectively. The summary attributable risk, considering the effect of all four variables together was 99%.

5.5 Discussion

Because false positive results can occur with any diagnostic test, we had more confidence that a herd was truly exposed as the proportion of animals testing positive increased. Therefore, by expressing each herd’s exposure to an infectious agent as a proportion of the sample that tested positive, we gave increasing weight to the diagnostic variables as our confidence in true herd exposure increased. This method also more fairly applied individual test results for a herd level diagnosis compared to assigning dichotomous (yes, no) exposure values when sample sizes varied between herds.31
Because WD outbreaks have been shown to cluster by time and geography\textsuperscript{24} we felt that the population of herds at greatest risk for winter dysentery were those herds in proximity to other outbreaks; therefore, herds were matched by time and geographical location. Conditional logistic regression considers the matched study design in the analysis. A disadvantage of matching is the potential to lose power to measure the true level of effect when the matching variable is not related to the exposure and to the disease.\textsuperscript{32} The OR estimations for the prevalence of BCV and BVDV titer increases were similar by either logistic regression or conditional logistical regression; but, the standard error of those statistics were greater with conditional logistic regression, indicating that the matching was inefficient.\textsuperscript{32} Therefore, the matching was disregarded and the OR's were estimated by logistic regression.

Case-control OR's represent the odds of exposure in diseased herds compared to the odds of exposure in control herds. For diseases that occur less frequently, such as WD, the OR is a reasonable estimate of the relative risk of a disease.\textsuperscript{33} Therefore, for purposes of discussion, the terms risk, or relative risk, will be used synonymous with the reported OR.

The risk for WD increased greatly as the prevalence of animals demonstrating an antibody response to BCV in a herd increased. This strong magnitude of effect contributes to the body of evidence implicating BCV as an important etiologic agent for WD.\textsuperscript{7,34} Before multivariable modelling, there was decreased risk for WD in herds with higher higher mean log\textsubscript{2} acute BCV antibody titers, suggesting that herds with
higher average BCV antibody titers may have been protected from WD. However, the acute BCV titer variable dropped out of the multivariable model when the apparently more informative BCV seroresponse prevalence variable was considered. The role that herd immunity to BCV might play in WD is not clarified by this study, since BCV seroresponses occurred in herds with a wide range of acute geometric mean titers, and the protective role of acute BCV IgG titers was not supported in the multivariable logistic models.

Logistic modeling identified several other variables that may be associated with WD outbreaks. Increased prevalence of animals in a herd demonstrating BVDV SN seroresponse was associated with increased risk for WD, though not at the same magnitude of effect as for BCV seroresponse. Herds with higher BVDV SN seroresponse prevalence tended to be different herds than those with high BCV seroresponse prevalence. It may be that some acute BVD outbreaks are clinically indistinguishable from WD.\textsuperscript{1,2} Previously, a prospective study of 9 New York dairies failed to demonstrate serologic evidence of an association of infectious bovine rhinotracheitis virus or BVDV with WD.\textsuperscript{35} Recently however, other researchers have suggested that BVDV may play a role in some WD outbreaks.\textsuperscript{15}

Several management related risk factors were associated with WD outbreaks. Increased stocking density was associated with greater risk for WD until the housing type variables were considered in the multivariable modelling. It is likely that housing density served as a confounder for the more informative housing type variable. Herds
where the cattle were housed in tie stalls or stanchion housing were at greater risk for WD than herds using freestall housing. A dose effect was apparent since the risk of WD among those farms where both types of housing existed was intermediate to the risk on those farms using either housing type alone. In typical tie-stall and stanchion barns cattle stand head-to-head, often with limited air space in front of them. This housing arrangement might facilitate transmission of aerosolized infectious agents, thereby increasing the risk of disease associated with these airborne agents. The risk of WD was also greater in herds that used manure handling equipment (e.g. skid steer loaders) to handle feed. Manure contamination of feed might serve as an efficient means of spreading enteric pathogens when those agents are present in the feces of some animals, facilitating a herd level outbreak of disease. The variables representing housing type, and use of manure handling equipment to handle feed have not previously been demonstrated to be risk factors for WD.

Even though this study demonstrated a significant strength of association between BCV and WD, consideration of the other explanatory variables identified by logistic modeling in this study, and the findings of others, suggests that WD may be a multi-agent, multifactorial disease. Taken in total, the etiologic and management risk factors identified in the 4-variable logistic model are logical considering what we know about these agents and the epidemiology of WD. Because BCV and BVDV are transmitted by both fecal-oral and respiratory routes, rapid spread by either of these
agents could be enhanced by close head-to-head confinement, or manure contamination of the feed.

Some risk factors identified in previous field investigations were not corroborated in ours. We did not measure a significant effect of herd size in this study, even though a large range of herd sizes were included in the study. Neither was recent (past 3 years) history of WD predictive of the WD outbreaks we studied. The power of this study, with 36 herds, was too low to detect any but very strong associations, so inferences about negative findings in this study may not be appropriate. The measured strength of association of a causal factor is dependent on the distribution of that factor, and related causal factors, in the population studied. Therefore, differences by geographical location, in the prevalence of infectious and non-infectious risk factors, may also explain the lack of consensus between this and previous WD risk factor studies. For example, it would be difficult to demonstrate that tie-stall barns were a risk factor for WD if few of the herds used tie-stalls in the population studied.

Estimates of attributable risk consider the impact of the risk factors identified on the disease experience of the population studied. Recent BCV exposure was attributable to an estimated 71% of the WD outbreaks in the population. Conversely, 29% of WD outbreaks in this population were not attributable to recent BCV exposure; so other agents or other risk factors may have played a causal role in WD, independent of BCV exposure. The exploratory risk factors, recent BVDV exposure, use of tie-stalls or stanchions compared to freestalls, and the use of manure handling
equipment to handle feed, all had meaningful attributable risks; and, if these variables are causally related to WD, then the presence or absence of each could have a large impact on the WD experience of the population studied. Considered together, the 4-variable logistic model could explain an estimated 99 % of the WD seen in the population of Ohio dairy herds studied. These factors deserve further investigation to confirm their role in WD outbreaks.
5.6 Acknowledgments

The authors thank Brian Winters for his assistance with DHIA records and the participating dairy farmers, veterinary practitioners, and county extension agents, who made this study possible. This report represents a portion of a dissertation submitted by the senior author to the Graduate School of The Ohio State University as partial fulfillment of the requirements for the PhD degree. This research was supported by grants from the American Veterinary Medical Foundation (#95-08), the Ohio Dairy Farmers Federation, the USDA, Special Animal Health Research Grants program (CSRS-89-34116-4548), and by state and federal funds appropriated to the Ohio Agricultural Research and Development Center (OARDC), The Ohio State University. This report is approved as OARDC article no. 31-97.
5.7 Footnotes

a. DHI Cooperative., Powell, OH

b. See Chapter 6.

c. Maxisorp Immunoplate, Nunc, Naperville, IL

d. ICN Biomedicals, Costa Mesa, CA

e. Difco, Detroit, MI
5.8 References


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infections in calves: Viral replication in the respiratory and intestinal tracts. *Am J Vet

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<th>BVDV Seroresponse</th>
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Table 5.1 -Results of diagnostic testing for 12 winter dysentery case and 24 control Ohio dairy herds. Bovine coronavirus (BCV) exposure was measured by ELISA to detect BCV antigen, and by ELISA to measure IgG antibodies seroresponse. Salmonella exposure was determined by selective fecal culture. Bovine viral diarrhea virus (BVDV) exposure was measured by serum neutralization seroresponse. A seroresponse for BCV and BVDV was defined as at least a 4-fold increase in the respective convalescent titer over the acute.

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<th>Control</th>
<th>Feces</th>
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* Case herd #7 and control herd #2 were the same herd at different points in time. † Case herd #30 was positive for BVDV by PCR of bulk tank milk. ‡ Adult herds BCV vaccinated at dry-off. § Herds not vaccinated against BVDV.
### Best univariable logistic model

-2 Log likelihood $X^2 = 14.576$, 1 df, $p < 0.0001$

<table>
<thead>
<tr>
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<th>Odds ratio</th>
<th>95% Conf. Int.</th>
<th>p-value</th>
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<tr>
<td>Each 10% BCV herd seroresponse</td>
<td>15.5</td>
<td>(1.672, 143.3)</td>
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<td>(Goodness of fit p-value = 0.4436)</td>
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### Best 2-variable logistic model

-2 Log likelihood $X^2 = 21.225$, 3 df, $p < 0.0001$

<table>
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<tr>
<td>Stanchion or tie stalls</td>
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<tr>
<td>Both stanchion and freestalls</td>
<td>5.6</td>
<td>(0.217, 145.7)</td>
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<td>(Goodness of fit p-value = 0.8233)</td>
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### Best 3-variable logistic model

-2 Log likelihood $X^2 = 21.336$, 3 df, $p < 0.0001$

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<td>(1.315, 128.6)</td>
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<tr>
<td>Each 10% BVDV herd seroresponse</td>
<td>3.5</td>
<td>(1.018, 11.8)</td>
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<tr>
<td>Handle feed with manure equipment</td>
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<td>(0.783, 113.7)</td>
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<td>(Goodness of fit p-value = 0.5052)</td>
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### Best 4-variable logistic model

-2 Log likelihood $X^2 = 26.713$, 5 df, $p < 0.0001$

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**Table 5.2** - Best (by -2 Log likelihood $X^2$ values) 1-4 variable logistic regression models to explain the occurrence of winter dysentery in 12 case and 24 control Ohio dairy herds investigated during the 1992-1993 and 1993-1994 winter seasons.
Figure 5.1 - Frequency distribution for bovine coronavirus (BCV) seroresponse among 12 winter dysentery case and 24 non-affected control Ohio dairy herds during the winter seasons of 1992-93 and 1993-94. A BCV seroresponse was defined as at least a 4-fold increase in the convalescent BCV IgG antibody ELISA titer over acute titer values.
Figure 5.2 - Univariable odds ratios with 95% confidence intervals showing, for each variable, the odds of exposure in 12 winter dysentery (WD) herds to the odds of exposure in 24 non-affected herds. Odds ratios greater than 1 indicate an association of the variable with WD, odds ratios less than 1 indicate an association of the variable with not having WD.
6.1 Summary

Objective: To identify potential risk factors to explain the occurrence of disease for individual adult cattle in herds experiencing winter dysentery (WD).

Animal population: Two hundred twenty-nine lactating and non-lactating adult cattle (125 cases and 104 controls) selected from within 12 WD affected Ohio dairy herds.

Procedure: Case-control study using multivariable conditional logistic regression to model the risk factors for disease in individuals, while adjusting for herd effects.

Results: The likelihood of disease increased as the titer of bovine coronavirus (BCV) antigen detectable in feces increased (OR= 2.94 for each 0.100 increase in BCV antigen ELISA value, p=0.031). Pregnant animals were less likely to be WD cases compared to non-pregnant herd-mates (OR= 0.49, p=0.016). Individuals seroresponding to BCV with high acute BCV antibody titers had with greater odds of disease compared to seroresponders with low acute titers. However, among those individuals not
seroresponding, high acute antibody titers were associated with lower odds of disease (p=0.013).

**Conclusions:** Within herds affected with WD, clinically ill cows were more likely to shed detectable titers of BCV antigen in the feces, and pregnancy appeared to be protective of disease. The measured interaction between BCV seroresponse, and acute BCV antibody titer levels may be evidence of an immunopathology, but could also be due to the dynamics of the test or study design.

**Clinical relevance:** The factors that explained a cow’s risk for illness within WD affected herds may have been surrogate measures for the individuals non-specific and BCV-specific immune profile.
6.2 Introduction

Winter dysentery (WD) is a disease syndrome characterized by diarrhea in adult beef and dairy herds. The onset of WD is acute, and the disease spreads rapidly within affected herds. Even though many individuals within an affected herd may become ill, mortality is typically low. In dairies the primary losses from WD are due to decreased milk production during and after the outbreak. Bovine coronavirus (BCV) has been incriminated as a causal agent of WD by researchers from around the world. The evidence incriminating BCV as an etiologic agent of WD is circumstantial, often based on isolation of the virus from WD affected individuals, or the demonstration of rising antibody titers to BCV in the serum of affected animals. In a study to assess the herd-level risk factors for WD, we found that herds with higher prevalences of animals demonstrating 4-fold or greater convalescent serum BCV IgG antibody titers had a great risk for WD. Results from that study also suggested that recent herd exposure to bovine viral diarrhea virus (BVDV), housing animals in tie-stall or stanchion barns compared to free-stall barns, or the use of manure handling equipment to handle feed might also have increased a herd’s risk for WD.

It is not clear why certain individual animals within WD affected herds get sick while other herdmates do not. Some researchers have suggested that heifers, high producing cows, and animals early in lactation may be at a greater risk for winter dysentery, but these observations have not been tested in controlled investigations. Better understanding of the cow-level risk factors for clinical disease in WD affected
herds would improve our understanding of WD and may help us define preventive measures. We report the results of a case-control study conducted within WD affected herds to identify infectious and non-infectious risk factors that explain why some cows, during WD outbreaks, become ill while other herdmates do not.

6.3 Materials and methods

**Source of animals.**

Case and control animals were selected from within 12 WD-affected Ohio dairy herds. The herds experienced WD during the winter months of 1992-93 and 1993-94. In these herds, WD was diagnosed when: 1) the primary clinical sign was diarrhea in adult cattle; 2) more than 15% of the adult herd, or a total of 15 animals, were affected; 3) most of the animals were ill within one week of the onset of clinical signs; 4) the case-fatality rate was less than 2% during the outbreak; and 5) the outbreak occurred between November 1 and April 30.

**Definition and selection of cases.**

After a case herd was identified, the dairyman was asked to prepare a list of the adult animals that were observed to have diarrhea. From this list approximately 10 animals were selected for sampling. If 10 or fewer animals were observed with diarrhea then all of the affected animals were sampled. If the list of diarrheic animals contained only slightly more than 10, the 10 most recently affected animals were sampled. If the list was longer, then 10 animals were systematic-randomly selected for sampling based on the last digit of the animals identification number.
Definition and selection of controls.

The dairyman also prepared a list of control animals from the remaining adult herdmates that were observed not to have diarrhea or inappetence. Animals were not included on the list of controls if the dairyman could not determine their disease status with confidence. Ten animals were sampled from the list of controls using the same systematic-random selection method as cases. Animals selected as controls that subsequently developed diarrhea they were reclassified as cases and not replaced as controls.

Data collection.

Serum and feces were collected from cases and control animals as soon as possible after the onset of the outbreak. Convalescent serum was collected from the same animals approximately 3 weeks later. Feces were kept on ice or refrigerated, and aliquoted within 24 hours of collection. During aliquoting, fecal samples were subjectively scored 0-4 for diarrhea consistency (0 being normal and 4 completely fluid). Fecal samples were submitted on ice for bacterial culture, and the remaining aliquots were frozen at -70 C until BCV diagnostics were performed. Feces from each animal were tested by BCV antigen capture ELISA, and Salmonella culture. The BCV antigen capture ELISA results were quantified as an ELISA value (signal OD minus background OD). An ELISA value $\geq 0.100$ was considered positive for BCV antigen. Salmonella culture results were reported as positive or negative after serotyping.
All paired sera were tested by ELISA to detect BCV IgG antibody responses, and by serum neutralization assay (SN) for bovine viral diarrhea virus antibody (BVDV) responses. Both of these serum assays were conducted using serial 2-fold dilutions. Seroresponses for either serum assay were defined by a minimum of a 4-fold increase in the antibody titer.

Subsets of the fecal samples were examined by BCV specific immunoelectron microscopy (IEM) and a subset of paired serum samples were tested by BCV serum neutralization to validate the results of the BCV antigen and antibody detecting ELISAs. Three to 4 fecal samples from each herd were cultured for *Campylobacter* spp. Data from recent medical and production records of cases and control animals were collected and tabulated. Heifers were defined as first lactation or dry (non-lactating) adult animals prior to a second lactation. Pregnancy status was dichotomized from the herd records as confirmed pregnant, or not pregnant. Animals were classified as lactating or dry (not lactating). Last date of calving was recorded for lactating animals, and date due to calve was recorded for dry cows. The number of days in milk at the time of the outbreak (DIM), was calculated by subtracting the date of last calving, or expected parturition date, from the date of the outbreak and was then categorized as less than 22 days, 22 to 99 days, and at least 100 days of lactation or non-lactating. Dairy Herd Improvement Association (DHIA) records from the monthly test prior to the outbreak were collected for the animals in herds where this information was available. Each individual’s DHIA relative-value was dichotomized as
less than 100, or 100 and greater, and also categorized as less than 95, 95-105, and >105.

Statistical methods.

Results from the diagnostic assays, and data from health, and production records were tabulated in a computer spreadsheet program as independent variables. Categorical variables were converted to design (dummy, 1,0) variables. Titer values were log₂ transformed before being tested in any statistical procedures. Group differences were compared using non-parametric statistics. Sensitivity, specificity, and kappa values were calculated to measure agreement between IEM and BCV diagnostic assays using epidemiologic computer software.

Odds ratios (OR), and 95 percent confidence intervals were calculated from conditional logistic regression coefficients using the herd of origin as a stratifying variable to adjust for lack of independence between individuals within herds. Variables from DHIA information were not considered for multivariable modeling because these data were only available from a subset of the study animals. Conditional logistic models were built by manual forward variable selection and backwards elimination. Variables contributing significance to the -2 log likelihood $X^2$ at p-values $\leq 0.05$ were kept in the model. After the significant explanatory variables were identified, we tested 4 interaction terms in the model at a -2 log likelihood p value $\leq 0.05$: 1) heifer status and number of days in milk, 2) BCV and BVDV seroresponse, 3) acute BCV IgG antibody titer (log₂ transformed) and whether there was a BCV seroresponse, and 4)
acute BVDV SN titer (log$_2$ transformed) and whether there was a BVDV seroresponse.

6.4 Results

We visited 12 herds affected with WD during the 1992-93 and 1993-94 winter seasons. The cumulative incidence of diseased individuals in these herds ranged from 15 percent to 100 percent. Ten herds had at least some DHIA data. The median adult herd size was 57 cows, the range was 25 to 225. Five of the herds were housed in free-stalls exclusively, 5 in tie-stalls, or stanchions, and 2 farms housed their cattle in both free-stalls and tie-stall (or stanchion) housing. All of the herds vaccinated at least annually against BVDV.

The outbreaks were clinically apparent for a median of 6 days (range 3-11). The median time from the onset of the outbreak until we collected acute diagnostic samples was 3 days (range 1-9); convalescent samples were collected at a median of 28 days later (range 20-37). Data was collected from 229 animals: 125 cases and 104 controls. Four case and 1 control animals were culled or died between acute and convalescent sampling. Seven cases and 8 controls were non-lactating animals. One hundred seventeen cases and 99 controls were Holstein. At the acute sampling the consistency of the feces was normal from 36 case and 83 control animals (fecal score=0), softer than normal from 43 cases and 17 controls (fecal score=1) and liquid from 46 cases and 4 controls (fecal score ≥2). Pregnancy had been confirmed in 49 of 123 cases and 56 of 104 controls.
Salmonella spp. was cultured from the feces of 10 individuals (4 cases and 6 controls) representing 2 farms. Only 1 animal, a control, was positive for Campylobacter upsaliensis of 82 (71 cases, 11 controls) animals cultured randomly from the 12 herds.

Eleven individuals (8 cases and 3 controls) from 3 farms were positive for BCV antigen in the feces by ELISA. Cases had a median BCV antigen capture ELISA value of 0.024 (range: -0.095 to 0.585), and the median among controls was 0.018 (range: -0.050 to 0.118). A BCV seroresponse was measured in 39 individuals (24 cases and 15 controls) from 9 herds. The geometric mean acute BCV IgG antibody titer among cases was 1275 (range: <50 - 21,527), and among controls was 1538 (range: <50 - 24,285). The adult cows in 1 herd were vaccinated against BCV during their dry period. The median acute BCV titer from the 20 individuals sampled in that herd was higher than the median acute BCV titer for the 204 individuals from the remaining unvaccinated herds (Wilcoxon rank sum p = 0.011); and 1 individual from that herd demonstrated a BCV IgG antibody seroresponse.

A BVDV seroresponse was measured in 19 individuals (7 cases and 12 controls) from 7 farms. The geometric mean acute BVDV serum neutralization titer among cases was 14.3 (range: <4 - 256), and among controls was 16.1 (range: <4 - 256). Four herds had both BCV and BVDV seroresponses, but only 1 individual seroresponded to both agents.
The feces from 77 animals, 62 cases and 15 controls, from within WD affected herds, were examined by IEM to detect BCV particles. The sensitivity and specificity of the BCV antigen capture ELISA compared to IEM was 0.42 and 1.00 respectively. The level of agreement beyond chance between these two assays was represented by a Kappa value of 0.50 (p <0.0001, Table 6.1).

Seroresponse to BCV, as determined by IgG antibody ELISA, had moderate agreement with presence of BCV antigen in feces as determined by IEM (Kappa = 0.57, p <0.0001) and had better agreement with IEM results than did BCV seroresponse as determined by BCV serum neutralization assay (Kappa = 0.31, p=0.09). However, when the results of BCV ELISA seroresponse and IEM were stratified by the acute BCV IgG antibody titer value, the level of agreement differed by strata (Table 6.2), having less agreement at titer levels below 1100 (Kappa =0.40) and greater agreement at titer levels above 1100 (Kappa =0.84). Further, the type of disagreement between IEM and demonstration of a BCV seroresponse also differed by these strata (Table 6.2). From the 28 individuals with acute BCV titer values 1100 and above, the tests did not agree for 1 IEM positive but non-seroresponding individual. From the 46 individuals with acute titer values below 1100, the tests did not agree for 15 IEM negative but seroresponsive individuals. There was no significant difference in the magnitude of seroresponse between cases and controls, either among the 15 seroresponders with negative IEM findings (Wilcoxon rank sums p=0.52), or among the total 39 total seroresponders (Wilcoxon rank sums p=0.19).
The univariable conditional logistic regression models for the factors measured are summarized (Table 6.3). The significant univariable factors to explain clinical disease during WD outbreaks were confirmed pregnancy status (OR=0.464 if confirmed pregnant), days in milk at the time of the outbreak (OR=2.729 if 0-21 days in milk, and OR=2.026 if 22-100 days in milk, compared to animals 100 days in milk or non-lactating), and the fecal BCV antigen capture ELISA value (OR= 2.436 for each 0.100 increase). Univariable ORs for the factors available from those herds on DHIA test are summarized (Table 6.4). Case individuals were much more likely to have soft or loose feces at the time of acute sampling than controls (OR =6.601 for fecal scores of 1 and 29.021 for fecal scores of 2 or more, p<0.0001).

Complete data were available for multivariable modelling from 224 individuals (121 cases and 103 controls). The factors that explained clinical disease in an individual, after multivariable modeling, were the fecal BCV antigen-capture ELISA value, pregnancy status, and an interaction between the acute BCV log₂ titer and the occurrence of a BCV seroresponse (Table 6.5). After adjusting for the other variables in the model, each increase of 0.100 in the BCV antigen ELISA value was associated with 2.94 times greater risk of being a diarrheic animal. Pregnant animals were only 0.49 times as likely to have diarrhea as their non-pregnant herd-mates. Animals that did not serorespond had decreasing odds of diarrhea if they had higher acute BCV IgG antibody titers; however, for seroresponders the odds for diarrhea increased with higher acute BCV antibody titers (Figure 6.1).
6.5 Discussion

By using a case-control study design we compared the differences between WD affected individuals with their non-affected herd-mates to make inferences about why some animals become ill in WD affected herds whereas others do not. However, inferences drawn from case-control studies are susceptible to biases from study design, and analysis. Misclassification bias can occur by disease, or by exposure. In this study errors in disease classification could occur at two levels: by herd, or by individual. No diagnostic assay exists for WD, but misclassification of disease at the herd level was minimized by use of a strict herd definition of WD. Also, each case herd was visited by the primary investigator (DRS) during the outbreak to help assure diagnostic uniformity. The dairy farmers were asked to classify individuals in their farms as affected (cases) or non-affected (controls). Certainly the individual disease classifications were based on subjective evaluation, defining diarrhea in cattle is not clear-cut, nor is appetite; however, the calculated OR's for fecal scores show that soft and liquid stool were strongly associated with case individuals; suggesting that the disease classifications were accurate.

An individual's exposure to BCV was measured using 2 recently developed ELISAs. The BCV antigen-capture ELISA proved to be very specific, but was not very sensitive for BCV detection compared to IEM. The poor sensitivity of this ELISA when testing adult cow feces was disappointing in view of the accuracy the assay demonstrated with diarrheic calf feces, and might be due to copro-antibodies in
adult cow feces complexing with BCV antigen and blocking the ELISA reaction. However, in spite of the poor sensitivity of the assay we were still able to demonstrate a significant association between the presence of BCV antigen in the feces and being a case. Because we expect the lack of test sensitivity to be non-differential between cases and controls, the OR’s we estimated for increasing BCV antigen capture ELISA values may have been an underestimate of the true effect.

A number of animals with BCV seroresponses did not have evidence of BCV particles in their feces by IEM. This type of disagreement could be due to lack of sensitivity of IEM because of failure to detect low numbers of virus particles present (titers of $\geq 10^5$ PFU/ml are usually required to detect virus), or because the window of virus shedding in the feces was missed due to sample collection timing. Alternatively, the disagreement could be due to lack of specificity of the BCV IgG antibody seroresponse. To assess whether the disagreement between BCV seroresponse and IEM results was differential between cases and controls, we compared the magnitude of the BCV seroresponse between the 9 cases and 6 controls that seroresponded but were IEM negative. The magnitude of BCV seroresponse was defined as the difference between the log₂ acute and convalescent titers, which is equivalent to the number of dilutions difference between acute and convalescent serum endpoints in the ELISA. We assumed that a greater magnitude of seroresponse gave us greater confidence in the result. Since the magnitude of the BCV seroresponse was not different between cases and controls either among the 15 seroresponding animals not in
agreement with IEM, or among all of the BCV seroresponding animals, we had equal confidence in the ELISA results regardless of case status; therefore, if misclassification of BCV seroresponses did occur at lower acute titer values, it was non-differential between cases and control, and we expect any resulting bias in the magnitude of effect to be towards the null.

One animal with BCV particles identified by IEM in the feces did not have a demonstrable BCV IgG antibody seroresponse. This disagreement might have been due to lack of specificity of IEM, but more likely was because presence of virus in the feces was not indicative of recent systemic exposure, as was measured by the BCV IgG antibody ELISA; or there was an undetectable (less than 4-fold titer change) BCV IgG seroresponse because of sample timing or an already high acute titer value. Further, the type of disagreement was dependent on the acute BCV IgG antibody ELISA titer value: the ELISA being less specific (or IEM being less sensitive) for individuals with low acute titer values, and less sensitive (or IEM less specific) with high acute titer values.

Several factors were not demonstrated to be associated with the occurrence of disease within WD affected herds. We did not find enough evidence that infection with Campylobacter spp. was a risk factor to continue the expense of culturing all of the individuals. This was an expected outcome; the causal role of Campylobacter spp. in WD has largely been ruled out.\textsuperscript{2,15,23,24} Salmonella infection, as defined by fecal culture, was not predictive of disease in the univariable or multivariable conditional logistic
models. Others have also failed to demonstrate Salmonella organisms in WD outbreaks; however, the use of ELISA to detect antibodies to *Salmonella* spp. may yield different results and should be considered.

An individual's age, indicated by the number of lactations, did not explain disease status within WD affected herds, nor was there a significant difference in the odds for disease between animals with one lactation (heifers) and older animals. Several researchers have speculated that young adult animals are at greater risk for WD illness than older adults; however, this relationship has not verified yet in a controlled investigation, and other investigations have also failed to demonstrate a difference in risk by age. Milk production parameters, measured or calculated DHIA, did not explain disease occurrence within WD affected herds contrary to the speculation of others. However, milk production data was available from only 75 cases and 74 controls from 8 herds, and that information could have been derived from testing as much as 1 month prior to the outbreak, so the power to observe production differences between case and control animals in this study may have been limited.

Neither the acute BCV IgG antibody log2 titer or demonstration of a BCV IgG antibody seroresponse were statistically associated with disease status within WD herds in the univariable conditional logistic models. However, there was a statistically significant interaction between these 2 variables and disease status within WD herds in the final multivariable model. This interaction may represent a biological response to BCV exposure. For example it may be that some animals that did not serorespond to
BCV may still have been exposed to the virus, but were able to neutralize virus with only a local (mucosal) immune response and without a measurable systemic response, and animals with higher acute BCV IgG titers may have been better able to more readily neutralize BCV before clinical signs became manifest. We expect animals with higher acute BCV titer to be more protected from disease; yet, for those animals that had a measurable systemic response (a BCV IgG antibody seroresponse), lower acute titers were associated with less likelihood of disease, and higher acute titers were associated with greater likelihood for disease. It is interesting to speculate that this statistical interaction may be evidence of an immunopathology such as antibody-dependent enhancement of infectivity. Antibody-dependent enhancement of infectivity is not known to occur with BCV; but, has been demonstrated for another coronavirus, feline infectious peritonitis virus (FIPV) and other viruses. Antibody-dependent enhanced infectivity is measured in vitro, but its clinical manifestation has been demonstrated by more rapid mortality, following FIPV re-challenge, among previously FIPV sensitized kittens. Regardless, the biology of this interaction cannot be clarified within this study because it is also possible that a BCV IgG seroresponse was more likely to be misclassified depending on the acute BCV IgG antibody titer value (less specificity at low acute titers). Thus, it may be that this statistical interaction is a reflection of the dynamics of the assay involved or timing of sample collection rather than a biological effect within the animal.
The BCV antigen-capture ELISA value of feces was a significant predictor of disease status as an unadjusted variable in a univariable conditional logistic model and in the final multivariable model in spite of its low sensitivity compared to IEM. The dichotomous variable for BCV antigen ELISA positive or negative, using an ELISA value cut-off of 0.100 was not significant, perhaps because information was lost by the categorization. We interpret the model to suggest that case animals had greater odds of shedding detectable (perhaps not antibody-complexed) BCV antigen, since the ELISA value should semi-quantitatively represent the amount of free BCV virus present in the feces. It is unclear if this is a causal association, since temporality is not established. However, its significance in the model and strength of effect contributes to the growing body of evidence for a causal role of BCV in WD.

In the univariable model, days in milk at the time of the outbreak, categorized as 0-21 days, 22-99 days, and 100 or more days of lactation or non-lactating, was a significant predictor of disease status. Cows early in lactation had greater odds of disease than cows 100 days or more in lactation. This variable was empirically categorized to fit biologically important time periods in a dairy cow’s life: the early post-partum period, the period characterized by increasing milk production and resultant loss in body condition, and the period characterized by decreasing milk production and return of body condition. This finding was consistent with the suggestion by other researchers that recent calving and early lactation may be important risk factors for WD.\textsuperscript{15,25,26,30} However, in the multivariable modeling process, the days
in milk variables lost statistical significance after adjusting for the effect of pregnancy status.

In the multivariable model, animals confirmed pregnant had less than one-half of the odds for disease compared to their non-pregnant herd-mates. Strict interpretation of the multivariable model would suggest that pregnancy status provided more information to explain disease status in WD herds than did days-in-milk. In fact, since it contributed the most to the -2 log likelihood $X^2$ value of the multivariable model, confirmed pregnancy status was the most explanatory factor for WD illness that we investigated.

Whether or not the pregnancy status of the individual provides a biological explanation for disease outcome must be considered carefully. The potential for confounding between pregnancy status and the categorical days-in-milk variables is high because they are nearly mutually exclusive and thus offer analogous (although inverse) information. Early lactation animals are rarely pregnant. The category of 0-21 days in milk is most exclusive of being pregnant, and had a higher measure of effect than did the category of 22-99 days in milk where some overlap with being pregnant might occur.

To our knowledge pregnancy has not been demonstrated to be a protective factor for infectious disease in domestic animals. The idea that pregnancy may be protective of disease should not be dismissed lightly since there is evidence that dramatic immunologic changes occur during pregnancy.\textsuperscript{31-37} The changes in an
individuals immunological profile during pregnancy are characterized by decreased specific immunity, and increased non-specific immunity. The increased non-specific immunity, characterized by increased phagocytic and complement activity, might be protective of infectious disease; or alternatively, if clinical signs of WD were due to an immunopathology, as the interaction term in the model suggests, then the decreased humoral responses that occur during pregnancy might be protective of disease following BCV exposure. Finally, it may be that both factors, stressors of early lactation and pregnancy status, have a role to play in the occurrence of disease during WD outbreaks.

Considered together, the variables in the multivariable conditional logistic model may have served as surrogate measures for non-specific immunity (pregnancy status), or BCV-specific immunity (shedding of free BCV antigens in the feces, and BCV IgG antibody responses). In the population of dairies herds studied, exposure to BCV was an important risk factor explaining a herd’s risk for WD outbreaks. It may be that, in this population, the reason that some cows got sick, while other herdmates did not, was best explained by the individuals BCV-specific, and non-specific immune profile.
6.6 Acknowledgments

The authors thank Brian Winters for his assistance with DHIA records and the participating dairy farmers, veterinary practitioners, and county extension agents, who made this study possible. This report represents a portion of a dissertation submitted by the senior author to the Graduate School of The Ohio State University as partial fulfillment of the requirements for the PhD degree. This research was supported by grants from the American Veterinary Medical Foundation (#95-08), the Ohio Dairy Farmers Federation, the USDA, Special Animal Health Research Grants program (CSRS-89-34116-4548), and by state and federal funds appropriated to the Ohio Agricultural Research and Development Center (OARDC), The Ohio State University. This report is approved as OARDC article no. 30-97.
6.7 Footnotes

a. See Chapter 5.

b. DHI Cooperative, Powell, OH

c. Epi-Info, Version 6.02, Center for Disease Control and Prevention, Atlanta, GA

d. PHREG procedure, SAS Institute Inc., Cary NC

e. Scourguard 3(K), SmithKline Beecham, Exton, PA
6.8 References


Table 6.1 - Measure of agreement between a bovine coronavirus antigen-capture ELISA and immunoelectron microscopy of fecal samples from 77 affected and unaffected individuals within 12 winter dysentery herds.
Table 6.2 - Comparison of the agreement between at least a 4-fold increase in bovine coronavirus (BCV) IgG antibody titer (seroresponse) and demonstration of BCV particles in the feces by immunoelectron microscopy for individuals within 12 winter dysentery affected herds. The comparison was made over all 74 individuals (A) with information from both assays, and after stratification by acute BCV IgG antibody titer (B and C).
<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds Ratio</th>
<th>Lower</th>
<th>Upper</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cows known to be pregnant</td>
<td>0.467</td>
<td>0.264</td>
<td>0.827</td>
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<tr>
<td>Each 0.100 rise in the BCV Ag ELISA</td>
<td>2.436</td>
<td>0.947</td>
<td>6.271</td>
<td>0.0233</td>
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<tr>
<td>Days-in-milk at the outbreak (compared to ≥100 days-in-milk or non-lactating)</td>
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<td></td>
<td></td>
<td>0.0339</td>
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<td>0-21 days-in-milk</td>
<td>2.729</td>
<td>0.900</td>
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<td>22-99 days-in-milk</td>
<td>2.026</td>
<td>1.053</td>
<td>3.897</td>
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</tr>
<tr>
<td>Days in milk at outbreak (continuous)</td>
<td>0.998</td>
<td>0.995</td>
<td>1.001</td>
<td>0.1561</td>
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<td>Log₂ of the acute BVDV SN titer</td>
<td>0.898</td>
<td>0.762</td>
<td>1.058</td>
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<td>Holsteins compared to other breeds</td>
<td>0.454</td>
<td>0.126</td>
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<td>0.2207</td>
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<td>Number of lactations</td>
<td>0.902</td>
<td>0.755</td>
<td>1.077</td>
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<td>Lactating animals compared to dry cows</td>
<td>2.057</td>
<td>0.574</td>
<td>7.379</td>
<td>0.2573</td>
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<td>BVDV SN seroresponse</td>
<td>0.567</td>
<td>0.206</td>
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<td>Log₂ of the acute BCV IgG titer</td>
<td>0.914</td>
<td>0.767</td>
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<td>0.3084</td>
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<tr>
<td>Positive fecal BCV antigen ELISA</td>
<td>1.515</td>
<td>0.285</td>
<td>8.060</td>
<td>0.6232</td>
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<td>Any health problems in the last 30 days</td>
<td>1.294</td>
<td>0.455</td>
<td>3.681</td>
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<td>Heifers compared to older cows</td>
<td>1.129</td>
<td>0.627</td>
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<td>Positive <em>Salmonella</em> culture</td>
<td>0.691</td>
<td>0.083</td>
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<td>BCV IgG antibody seroresponse</td>
<td>0.991</td>
<td>0.371</td>
<td>2.645</td>
<td>0.9851</td>
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</tbody>
</table>

BCV = bovine coronavirus, BVDV = bovine viral diarrhea virus, SN = serum neutralization

**Table 6.3** - Univariable odds ratios, 95% confidence interval, and -2 log likelihood $X^2$ p-values from conditional logistic regression to explain diarrhea among 229 adult cows in 12 winter dysentery herds investigated during the 1992-1993 and 1993-1994 winter seasons.
Table 6.4 - Univariable odds ratios, 95% confidence interval, and -2 log likelihood $X^2$ p-values from conditional logistic regression to explain diarrhea among the cows with DHIA test data available in winter dysentery herds investigated during the 1992-1993 and 1993-1994 winter seasons. The DHIA information was not available from all herds so these variables were not considered during multivariable modelling.
<table>
<thead>
<tr>
<th>Variable</th>
<th>β parameter</th>
<th>Odds ratio</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Each 0.100 rise in the BCV Ag ELISA</td>
<td>1.079</td>
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<td>0.0305</td>
</tr>
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<td>Cows known to be pregnant</td>
<td>-0.723</td>
<td>0.49</td>
<td>0.0161</td>
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<td>Interaction terms</td>
<td></td>
<td></td>
<td>0.0310</td>
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<tr>
<td>Log₂ acute BCV IgG ELISA titer</td>
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<td></td>
</tr>
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<td>BCV IgG antibody seroresponse</td>
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<td></td>
</tr>
<tr>
<td>Interaction of both variables</td>
<td>0.999</td>
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<td></td>
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</tbody>
</table>

Model -2 log likelihood $X^2$ p-value = 0.001

BCV = bovine coronavirus

Table 6.5 - Multivariable conditional logistic model to explain the risk of diarrhea among the 224 cows with complete data, in 12 winter dysentery herds investigated in Ohio during the 1992-1993 and 1993-1994 winter seasons.
Figure 6.1 Illustration of the interaction between an individual's acute bovine coronavirus (BCV) IgG antibody level and BCV IgG seroresponse to explain an individual's odds for diarrhea within winter dysentery affected herds.
VALIDATION OF AN ANTIGEN-CAPTURE ELISA AND AN ANTIBODY-CAPTURE ELISA FOR USE IN DETERMINING BOVINE CORONAVIRUS EXPOSURE IN CAUSAL INVESTIGATIONS OF WINTER DYSENTERY

7.1 Summary

Objective - To validate an antibody capture ELISA for measuring bovine coronavirus (BCV) exposure in cattle. To explain the apparent loss of sensitivity of a BCV antigen-capture ELISA when testing adult cow feces rather than the feces of neonatal calves.

Animals - Subsets of diagnostic samples from adult cows in winter dysentery affected and non-affected dairies, and BCV-inoculated, or control, gnotobiotic and colostrum-deprived calves.

Procedures - Comparison between BCV serum antibody titers determined by an IgG antibody-capture ELISA and a plaque-reduction virus neutralization assay and their agreement with IEM detection of BCV in feces of the same individuals. Colostrum-deprived and gnotobiotic calves were inoculated with feces suspected to contain BCV as an \textit{in vivo} confirmatory test for infectious BCV. An ELISA was developed to detect BCV antigen-antibody complexes in feces for comparison with the results of the BCV antigen-capture ELISA and IEM.
**Results** - The BCV antibody capture ELISA correlated with BCV neutralization assays, but the ELISA results agreed more closely with IEM identification of viral particles in the feces. The reduced sensitivity of the antigen-capture ELISA was confirmed, because calves were infected by BCV ELISA negative adult fecal samples. The BCV antigen-antibody complex capture ELISA correlated with IEM and the antigen-capture ELISA.

**Clinical implications** - In adult cattle, testing of paired serum by the BCV antibody-capture ELISA may be a better indicator of recent BCV exposure than BCV neutralization tests. In adult cattle BCV antigen-antibody complexes in the feces may complicate the detection of BCV fecal shedding by ELISA.
7.2 Introduction

Our causal investigations of herd-level and cow-level risk factors for winter dysentery (WD) were summarized in Chapters 5 and 6. In these field investigations, exposure to bovine coronavirus (BCV) was assessed by the use of 2 assays: 1) an antigen capture ELISA to detect BCV antigens in the feces (BCV Ag ELISA), and 2) an ELISA to measure serum IgG antibody responses to BCV (BCV IgG ELISA).

The agreement between a ≥4-fold BCV IgG ELISA seroresponse and identification of virus particles in the feces was moderate and differed by the level of the acute BCV IgG ELISA titer (Chapter 6). The BCV Ag ELISA demonstrated excellent sensitivity and specificity when evaluated using diarrheic neonatal calf feces, but was less sensitive when testing adult cow feces compared to immunoelectron microscopy (IEM)(Chapter 6). Because of the nearly ubiquitous presence of BCV on farms and the fact that most adult animals are seropositive to BCV, it is probable that adult cattle respond rapidly to secondary BCV infections. Bovine coronavirus antigen-antibody complexes have been demonstrated in the feces of adult cattle. If BCV epitopes were saturated by copro-antibodies they would be undetectable by the ELISA methodology employed, but the virus particles could still be visible by electron microscopic methods. We hypothesized that the saturation of BCV antigens by copro-antibodies may be responsible for the apparent lack of sensitivity of the BCV Ag ELISA when testing fecal samples from adult cattle.
Further evaluation of these assays is useful to better understand the limitations of the assays, and perhaps aid in understanding the pathogenesis of WD. The objectives of this study were 2-fold: 1) to evaluate the agreement of the BCV IgG ELISA with other methods of BCV serology, and to compare the results of these serologic assays with the detection of BCV in the feces by IEM; and 2) to investigate the role of antigen-antibody complexes in the loss of sensitivity of the BCV Ag ELISA when testing adult cow feces.

7.3 Materials and methods

Comparison of serological assays

Paired serum samples from 469 adult cattle were tested for IgG seroresponses to BCV by antibody capture ELISA as described in Chapter 6. From the same individuals, 21 serum pairs were tested by a plaque reduction virus neutralization assay (BCV-SN) previously described. Ninety-two fecal samples from the 469 individuals were examined for BCV by IEM as previously described. Test results for all three assays were available for 15 individuals. Outcomes of the serologic assays were reported in 2 ways: 1) as the magnitude of the difference between the \( \log_2 \) convalescent titer and the \( \log_2 \) acute titers; and 2) as the dichotomous outcome of seroresponding, or not. Seroresponse by the ELISA was defined as a \( \geq 4 \)-fold increase in the convalescent titer over the acute, while seroresponse by SN was defined first as a \( \geq 4 \)-fold, and then \( \geq 2 \)-fold, increase in the convalescent titer.
The agreement between the response magnitude for the 2 serological assays was assessed by Pearson’s correlation and Spearman’s rank correlation. The agreement between a 4-fold ELISA seroresponse and either a 2-fold or 4-fold SN seroresponse was assessed with the kappa statistic (K). The kappa statistic was also used to compare agreement between the serologic assays and detection of BCV in the feces as determined by IEM.

*Calf inoculation trials.*

Gnotobiotic (GN), or colostrum deprived (CD) calves were inoculated orally and intranasally with 20% suspensions of feces in cell-culture media, as previously described. Fecal samples used for the inoculations were samples from the same WD affected individuals as described above, and defined by one of 2 criteria: 1) BCV Ag ELISA positive samples from various WD affected herds; or 2) BCV Ag ELISA negative, but IEM positive feces from individuals demonstrating a BCV IgG ELISA seroresponse. Feces from the calves were collected daily and their consistency monitored. Pre-inoculation feces from each calf served as controls. At, or shortly after the onset of diarrhea, the calves were euthanized. Feces or intestinal contents from the calves were examined by the BCV Ag ELISA, and subsets by IEM. Smears of intestinal mucosa cells were examined by a fluorescent antibody assay (FA) as described previously.
Bovine coronavirus antigen-antibody complex ELISA

Ninety fecal samples with IEM results from the 469 cows previously described were tested by an indirect ELISA designed to identify the presence of partially saturated BCV antigen-antibody complexes.

The BCV antigen-antibody ELISA (Ag-Ab ELISA) utilized a pool of 3 MAbs directed against 3 structural BCV proteins (N, S, HE) as positive coating in carbonate-bicarbonate buffer (pH 9.6). One hundred microliters of a 1:8000 dilution of the 3 MAbs, used as mouse ascites fluids, was added to paired rows as positive coating. Similarly, 100 μl of a 1:8000 dilution of BCV antibody-negative mouse ascites fluids was added to paired rows as negative coating. Plates were incubated at 4°C overnight. After washing, 200 μl of 5% non-fat dry milk in phosphate buffered saline, pH 7.4, (PBS) was added to each well as a blocking step to minimize non-specific binding and the plates were incubated for 2 hours at 25°C. The plates were washed and 100 μl of 1:25 dilutions of the test samples, in PBS, were placed in paired BCV antibody-positive and -negative ascites coated wells. Plates were incubated at 25°C for 1 hour. The plates were washed and 100 μl of rabbit anti-bovine IgG peroxidase-conjugated antibody diluted 1:1000 in PBS / 0.05% Tween 20 was added to each well. Plates were incubated for 1 hour at 25°C, and washed. One hundred microliters of the chromogen substrate, a 1:1000 solution of H₂O₂ and 2,2'-azino-bis(3-ethyl-benzthiazoline)sulfonic acid in 0.1M sodium citrate was applied to each well. The color reaction was stopped at 30 minutes, by the addition of 50 μl of 5% sodium
dodecyl sulfate per well. The absorbance value (OD) of each well was read at a wavelength of 414 nm. At each washing, the plates were rinsed 5 times with PBS/0.05% Tween 20. Plates were sealed with ELISA plate tape during each incubation step. PBS/0.05% Tween 20 was used on each plate as a negative control.

The outcome of the Ag-Ab ELISA was expressed as an ELISA value, which was the mathematical difference between the average OD value of the 2 positive serum coated wells and the average OD value of the 2 negative serum coated wells for each sample.

The Ag-Ab ELISA values from IEM positive samples were compared to the ELISA values from IEM negative samples by the Wilcoxon rank sums test. The predictability of the ELISA values for the dichotomous outcome IEM positive or negative was tested using logistic regression, before and after adjusting for whether or not the sample originated from a WD affected, or non-affected herd.

Agreement between the Ag-Ab ELISA and the Ag ELISA was tested using Ag ELISA values as continuous and dichotomous variables. The correlation between Ag-Ab ELISA values and the continuous BCV Ag ELISA values was tested by the Spearman rank correlation statistic. The predictability of the Ag-Ab ELISA values for the dichotomous outcome of Ag ELISA positive or negative at a cut-off ELISA value of 0.100 was tested using logistic regression, before and after adjusting for whether or not the sample originated from a WD affected, or non-affected herd.
7.4 Results

Comparison of serological assays

The magnitudes of the responses demonstrated by the two serum antibody assays were correlated (Pearson $r=0.71$, $p=0.0003$; Spearman rank $r=0.69$, $p=0.0006$). The linear regression equation, with a coefficient of determination ($r^2$) of 0.50, $p=0.0003$, was:

$$\text{Magnitude of the log}_2 \text{ ELISA response} = 1.40 + 0.82 \times \text{magnitude of the log}_2 \text{ SN response}$$

Bivariate plots illustrate the correlation between the response magnitudes between the 2 serological assays and the relative agreement with IEM examination of the feces from the same individuals (Fig. 7.1).

Agreement on seroresponse outcomes between the 2 serum assays was poor if a seroresponse by SN was defined as a 4-fold or greater response ($K=0.30$, one sided $p=0.027$). Agreement on seroresponse outcome was moderate between the 2 serum assays when seroresponse by SN was defined as a 2-fold or greater response ($K=0.53$, $p=0.005$).

The BCV IgG ELISA seroresponse outcomes demonstrated good agreement with IEM results ($K=0.626$, $p<0.0001$, Table 7.1). Seroresponse outcomes by SN showed poor agreement with IEM results regardless of how a SN seroresponse was defined (4-fold or greater SN response $K=0.31$, $p=0.09$, Table 7.2; 2-fold or greater SN response $K=0.34$, $p=0.09$, Table 7.3).
Calf inoculation trials

Eight BCV Ag ELISA positive fecal samples or 2 BCV Ag ELISA negative, IEM positive fecal samples from BCV seropositive cows were inoculated into 10 CD or GN calves, 30 to 60 days of age, as summarized (Table 7.4). The strength of the evidence for BCV infection varied for each calf, but all but 1 calf had at least one positive BCV test result and 7 of 10 had virus particles detectable by IEM in feces or intestinal contents.

Antigen-antibody complex ELISA

From the 90 fecal samples examined by IEM, BCV was positively identified in 24. The remaining 66 samples were considered negative for BCV, although coronavirus-like particles with shorter peplomers and without evidence of antibody clumping were demonstrated in 23 of these.

As illustrated (Fig. 7.2), the distribution of the ELISA values by the Ag-Ab ELISA were greater among the IEM positive samples than the IEM negative samples (Wilcoxon rank sum p=0.0454). Similarly, an association between increasing Ag-Ab ELISA values and the odds of finding BCV by IEM was demonstrated by logistic regression because each 0.01 increase in the ELISA value was associated with a 1.84 increase in the odds of being IEM positive (-2 log likelihood p=0.001). This measure of effect remained unchanged in a multivariate logistic model controlling for whether or not the samples originated from WD affected herds.
Higher Ag-Ab ELISA values tended to be associated with higher BCV Ag ELISA results. The ELISA values by the Ag-Ab ELISA were correlated with the ELISA values from the BCV Ag ELISA (Spearman rank correlation $r=0.21$, $p=0.043$), and by logistic regression, each 0.01 increase in the Ag-Ab ELISA value was associated with a 1.85 increase in the odds of being BCV Ag ELISA positive (-2 log likelihood $p=0.003$). This measure of effect remained unchanged in a multivariate logistic model controlling for whether or not the samples originated from WD affected herds.

7.5 Discussion

The magnitudes of the responses for both serologic assays were significantly correlated suggesting a tendency to measure similar effects. The assays measure greatly different immune responses (binding versus neutralization, and to different viral proteins), and the magnitude of those respective responses might be expected to vary. Literal interpretation of the $r^2$ suggests that 50% of the variability in the magnitude of the IgG response is predicted/explained by the SN response, and vice versa.¹⁰

Defining a dichotomous outcome of seroresponse resulted in less agreement between the two serum assays than did the magnitude of the response. The agreement was improved by changing the definition of a SN seroresponse from 4-fold or greater to 2-fold or greater.

If we assume that a seroresponse represents recent exposure to BCV, and that presence of BCV in the feces also represents recent exposure, then the serum assay that
best agrees with the IEM results may be the most predictive of BCV exposure. An ELISA seroresponse was more likely to be in agreement with IEM results than SN. And, when the ELISA and IEM disagreed, the disagreement was in the direction of not finding virus in the feces of individuals that had a seroresponse, as might be predicted: due to the brief, temporal, nature of virus shedding, it is plausible that virus might not be present in the feces of some individual that had a seroresponse.

Finally, SN outcomes varied in agreement with the other assays depending on whether an SN seroresponse was defined as a ≥2-fold, or ≥4-fold increase in the convalescent titer. Since adult cattle all appear to have acute SN titers it may be that the magnitude of the neutralizing response following exposure is not great enough to be predictive using the standard 4-fold or greater response definition. It may be that BCV SN, using a 4-fold or greater titer change to define a seroresponse, is not sensitive enough for use in adult cattle.

We were able to BCV-infect some, but not all, of the CD calves exposed to BCV Ag ELISA positive feces. This demonstration of the presence of infectious BCV in BCV Ag ELISA positive feces helps to validate those positive results. Some CD calves may not have been infected with BCV after exposure to these feces because the assay gave false positive results, virus antigen was present but not infectious, there was infectious virus present, but at in too low of numbers to cause detectable infection, or because the calves had already developed immunity because of prior, unplanned, exposure.
We also demonstrated the presence of infectious BCV particles in the feces of CD and GN calves exposed to BCV Ag ELISA negative feces that were IEM positive for BCV and from individuals demonstrating a BCV seroresponse. This finding confirmed that the BCV Ag ELISA lacked sensitivity compared to IEM as discussed in Chapter 6.

The Ag-Ab ELISA was designed to test our hypothesis that the lack of sensitivity of the ELISA might be due to copro-antibodies saturating antigenic sites on the virus and effectively blocking the ELISA reactions. The signal (increased ELISA value) from the Ag-Ab ELISA was relatively weak, but should represent bovine IgG antibodies specific to BCV antigens. The amount of signal generated by this ELISA should be dependent upon the relative amount of incomplete saturation of the virus antigens, because the virus-antibody complex can be measured only if enough virus antigenic sites are available to react with the BCV specific coating antibodies. Feces containing BCV as determined by IEM, or BCV Ag ELISA, should not produce a detectable signal with the Ag-Ab ELISA if there were no (or relatively few) antibodies complexed with the viral particles, or if the viral antigenic sites were completely saturated with antibodies, but should produce a signal with partial antibody complexing. Feces not containing BCV, as determined by IEM, should not produce a signal (within the limitations of the sensitivity of IEM) beyond background levels of the assay. The significantly elevated Ag-Ab ELISA values among the IEM positive fecal samples suggests the presence of partial antibody complexing of the BCV antigen present.
The results of BCV Ab ELISA and the Ag-Ab ELISAs were positively correlated and logistic models demonstrated increasing odds of the fecal sample being IEM positive or BCV Ag ELISA positive as the Ag-Ab ELISA signal increased. If BCV antigen-antibody complexes commonly occur in the feces of BCV infected adult cows then these findings are expected. When no virus is present, both ELISAs should produce only background levels of signal, and IEM results should be negative; but, as the amount of partially antibody complexed virus increases then both assays should increase in signal, and IEM results are more likely to be positive. Finally, as the virus becomes saturated with antibodies, both assays should decrease in signal.

Our findings support those of others that BCV specific antigen-antibody complexes do appear in the feces of adult cattle. If BCV antigens become saturated with antibody then antigen-capture ELISA reactions may be blocked. This may explain why some adult cow feces determined negative for BCV by antigen-capture ELISA had BCV particles detectable by IEM and were infectious to CD calves.

These investigations were undertaken to more fully understand the limitations of 2 BCV diagnostic assays we developed for use in observational and experimental research. Epidemiologists and diagnosticians rely on diagnostic assays to make valid inferences about exposure to infectious agents. It is important that we clearly understand what is measured with the assays we use and that we clearly understand when, how, and why misclassification may occur with those assays.
7.6 References


### Table 7.1. Demonstration of agreement between BCV IgG ELISA and IEM by 2x2 table.

<table>
<thead>
<tr>
<th>BCV IgG antibody seroresponse</th>
<th>Immunoelectron microscopy</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>positive</td>
<td>negative</td>
</tr>
<tr>
<td>yes</td>
<td>24</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>no</td>
<td>1</td>
<td></td>
<td>52</td>
</tr>
</tbody>
</table>

Kappa = 0.626 \quad p< 0.0001
### Table 7.2. Demonstration of agreement between BCV SN, BCV IgG ELISA and IEM by 2x2 tables, when SN seroresponses were defined as 4-fold or greater convalescent titer increases.

<table>
<thead>
<tr>
<th></th>
<th>Immunelectron microscopy</th>
<th></th>
<th>BCV IgG ELISA seroresponse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>positive</td>
<td>negative</td>
<td></td>
</tr>
<tr>
<td><strong>BCV serum neutralization seroresponse</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>yes</td>
<td>3</td>
<td>1</td>
<td>yes</td>
</tr>
<tr>
<td>no</td>
<td>4</td>
<td>7</td>
<td>no</td>
</tr>
</tbody>
</table>

Kappa = 0.312  p=0.09  
Kappa = 0.300  p=0.027
<table>
<thead>
<tr>
<th>BCV serum neutralization seroresponse</th>
<th>Immunoelectron microscopy</th>
<th>( p = 0.09 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>yes</td>
<td>positive: 5, negative: 3</td>
<td>( Kappa = 0.336 )</td>
</tr>
<tr>
<td>no</td>
<td>positive: 2, negative: 5</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BCV serum neutralization seroresponse</th>
<th>BCV IgG ELISA seroresponse</th>
<th>( p = 0.005 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>yes</td>
<td>positive: 8, negative: 1</td>
<td>( Kappa = 0.533 )</td>
</tr>
<tr>
<td>no</td>
<td>positive: 4, negative: 8</td>
<td></td>
</tr>
</tbody>
</table>

**Table 7.3.** Demonstration of agreement between BCV SN, BCV IgG ELISA and IEM, by 2x2 tables, when SN seroresponses were defined as 2-fold or greater convalescent titer increases.
<table>
<thead>
<tr>
<th>Log ID</th>
<th>Herd</th>
<th>Case</th>
<th>BCV Ag ELISA Value</th>
<th>Calf</th>
<th>IEM</th>
<th>FA</th>
<th>BCV Ag ELISA</th>
<th>Fecal Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS 1647</td>
<td>30</td>
<td>Y</td>
<td>0.110 (+)</td>
<td>CD 285</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>4</td>
</tr>
<tr>
<td>DS 1704</td>
<td>31</td>
<td>NA</td>
<td>0.134 (+)</td>
<td>CD 294</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>DS 1707</td>
<td>31</td>
<td>NA</td>
<td>0.149 (+)</td>
<td>CD 293</td>
<td>-</td>
<td>ND</td>
<td>+</td>
<td>3</td>
</tr>
<tr>
<td>DS 1781</td>
<td>33</td>
<td>Y</td>
<td>0.389 (+)</td>
<td>CD 291</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>4</td>
</tr>
<tr>
<td>DS 1791</td>
<td>33</td>
<td>Y</td>
<td>0.497 (+)</td>
<td>CD 286</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>0-1</td>
</tr>
<tr>
<td>DS 306</td>
<td>8</td>
<td>N</td>
<td>0.118 (+)</td>
<td>CD 288</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td>DS 777</td>
<td>15</td>
<td>NA</td>
<td>0.101 (+)</td>
<td>CD 295</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>DS 1146</td>
<td>22</td>
<td>NA</td>
<td>0.116 (+)</td>
<td>CD 289</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>DS 1797</td>
<td>33</td>
<td>Y</td>
<td>0.090 (-)</td>
<td>CD 300</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>4</td>
</tr>
<tr>
<td>DS 1787</td>
<td>33</td>
<td>N</td>
<td>-0.015 (-)</td>
<td>B 378</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 7.4 Summary of diagnostic results from colostrum deprived (calf = CD) or gnotobiotic calf (calf = B) bovine coronavirus (BCV) inoculation studies. Fecal inoculums were either BCV antigen-capture ELISA (BCV Ag ELISA) positive (+) samples, or BCV Ag ELISA negative (-), but immunoelectron microscopy (IEM) positive and from BCV seroresponding individuals. Direct immunofluorescent antibody assay = FA. Fecal samples originated from affected (case = Y) or unaffected (case = N) adult cattle from winter dysentery, or control herds (case = NA). Calf feces were subjectively scored 0-4 for consistency (0 = firm, normal; 4 = fluid). Test results were designated as either positive (+) if one or more daily sample was test positive or negative (-) if no tests were positive. ND = not done.
Figure 7.1. Plot of the magnitude of response in log₂ titer values between acute and convalescent serum from 21 adult cows by BCV serum neutralization and BCV IgG antibody ELISA. The cut-off points for ≥4-fold seroresponses are indicated by solid lines and marker symbols indicate the IEM finding for the feces of the same individual.
**Figure 7.2.** Box and whisker plot of the distribution of bovine antigen-antibody ELISA values for fecal samples that were positive (n=24), or negative (n=66), for bovine coronavirus particles by immunoelectron microscopy (IEM). The distributions of the ELISA values were different between the 2 groups (Wilcoxon rank sum p = 0.0454). The box represents the 25th. and 75th. percentile values, and whiskers (vertical lines) extend to the maximum and minimum values.
8.1 Introduction

In 1978, Campbell and Cookingham reviewed previous investigations of winter dysentery (WD) and speculated on the causal factors of the disease. They first discussed the possibility that WD might be a syndrome representing several clinically similar diseases rather than a distinct disease; or secondly, that the disease is not of an infectious nature. They then suggested another alternative:

Thirdly, the disease is caused by a microorganism. In this event we would suggest that the following best fits the present description of the condition. The agent, a virus or a hard to grow bacterium is brought into the herd by an immune, inapparent carrier or mechanically in feed or even on humans. Factors necessary for the establishment of an out-break include stress, close confinement and a number of susceptible non-immune animals. Once established, there is rapid spread throughout the herd probably by aerosols of the agent suspended in feces or droplets from the respiratory tract, entrance being gained through the mucosa of the mouth or the respiratory tract. After a transient viremia the agent produces mild effects on the respiratory tract and more severe affects on the gastrointestinal tract before it is re-excreted. Many animals in the herd are infected but young non-producing and old "immune" cattle show less severe clinical signs than do stressed, non-immune, lactating cows. Recovery is accompanied by some degree of acquired immunity but future outbreaks can occur when a carrier is available, the herd is stressed and it includes a substantial number of susceptible animals. (emphasis added)¹
The final word on causal relationships in WD has certainly not yet been written, but through our studies we have perhaps provided further evidence in support of some of these speculations.

We started our research armed with prior evidence supporting the hypothesis that bovine coronavirus (BCV) was causally associated with WD. Recognizing that causes can act at different levels of organization, we designed herd-level and cow-level case-control field investigations to test this hypothesis. Like previous researchers, we speculated that other risk factors may play a role in the occurrence of WD outbreaks and we made use of the case-control study design to help clarify these speculations. Thus our investigations of WD were both hypothesis testing and hypothesis generating studies.

8.2 BCV diagnostic assay validation

Before these investigations could be conducted efficiently it was necessary to design and evaluate assays that were reliable and could rapidly test a large number of samples. The enzyme-linked immunosorbant assay (ELISA) was chosen as the diagnostic methodology most likely to meet these requirements.

An ELISA utilizing a pool of 3 monoclonal antibodies for BCV antigen capture from feces (BCV Ag ELISA) was sensitive and specific for diarrheic neonatal calf studies, but was less sensitive when testing adult cow feces. Using immunoelectron microscopy (IEM), BCV could be demonstrated in some adult cow feces which tested BCV Ag ELISA negative: inoculation of colostrum-deprived or gnotobiotic calves with
such IEM BCV-positive fecal samples confirmed their infectivity. Bovine coronavirus specific antigen-antibody complexes have previously been demonstrated in the feces of normal adult cattle. We hypothesized that these particles may have been saturated with copro-antibodies, preventing their reaction in the BCV Ag ELISA. This hypothesis was tested by developing an ELISA to demonstrate partially saturated BCV antigen-antibody complexes. The signal generated by this assay was greater among IEM positive fecal samples than IEM negative samples, suggesting that BCV antigen-antibody complexes were present only when the virus particles were evident. The signal generated by the BCV antigen-antibody complex ELISA was correlated with the signal from the BCV Ag ELISA, suggesting that as the amount of detectable virus antigens increased so did the amount of detectable antigen-antibody complexes. This is logical because both ELISAs should measure partially antibody-saturated virus antigens. As anticipated, increasing BCV antigen-antibody ELISA signal was associated with IEM positive and BCV Ag ELISA positive fecal samples.

In spite of poor sensitivity when testing adult cow feces, the BCV Ag ELISA was specific compared to IEM results. Furthermore, we demonstrated that feces testing positive by the BCV Ag ELISA contained infective BCV by infecting colostrum-deprived calves with test positive fecal samples.

Demonstration of an increasing (e.g. a 2- or 4-fold) serologic response provides evidence of recent exposure to an agent. We designed an antibody-capture ELISA to demonstrate IgG antibody seroresponses to BCV in paired serum samples taken 3-4
weeks apart. Serum IgG antibody responses sometimes occur following gut epithelial cell infections after macrophages present ingested antigen to lymphoid cells in mesenteric lymph nodes which then circulate systemically, or after antigen is processed by systemic antigen-processing cells and distributed to systemic lymph nodes following damage to intestinal epithelium. Most adult cattle have serological evidence of previous exposure to BCV, and so we expected recent BCV exposure to result in a primarily IgG anamnestic seroresponse. This assay was validated by comparison of testing paired adult cow serums in a BCV plaque reduction virus neutralization assay (BCV-SN) and the demonstration of BCV virus particles in the feces of the same individuals by IEM. The magnitude of seroresponse by the antibody-capture ELISA correlated with the equivalent information from the BCV-SN assay, but demonstration of a ≥4-fold seroresponse had greater agreement with positive IEM findings than did either a ≥2-fold or ≥4-fold seroresponse by the BCV-SN assay. It is not clear why ≥4-fold seroresponses from individuals with acute BCV IgG antibody titers ≥1100 had greater agreement with IEM results than seroresponses from individuals with titers <1100, but this finding may relate to the timing of the acute sample collection relative to initial exposure to BCV and the window of fecal shedding of BCV.

8.3 Herd level investigation of winter dysentery

In the past there has been some confusion over the definition of WD. To minimize misclassification of disease, we used a specific herd-level definition of WD which addressed the class of animal, clinical signs, and placed limitations on morbidity
and mortality. Therefore, causal inferences derived from this study refer specifically to WD as we defined it.

Because a cow-level study was nested within the herd-level investigation, 2 potential problems with study design were addressed. First, diagnostic information was collected from an average of 20 individuals from case herds, but only 10 individuals from control herds. We avoided bias, due to differences in sample size between case herds and control herds, by reporting the herd level exposures in terms of the herd-prevalence of positive tests or geometric mean titers rather than reporting a dichotomous herd-level outcome of exposed or not exposed. Secondly, within each WD affected herd approximately equal numbers of diagnostic samples were collected from individuals selected from a list of sick individuals, and from a list of non-affected individuals; and from these 2 groups a herd prevalence of exposure was estimated. Therefore, the apparent prevalence of sick animals in each herd was approximately 50%, even though the true prevalence of sick animals ranged from 15% to 100%. This study design could lead to biases in the estimates of agent exposure prevalence unless the rate of exposure was similar among cases and controls. To test for potential bias, the prevalence of agent exposure was estimated three ways: 1) prevalence of exposure among only sick individuals tested in a herd; 2) prevalence of exposure among only well individuals tested in a herd; 3) prevalence of exposure among all individuals tested in a herd. Neither the univariable odd ratios nor the multivariable models for WD determined using each of these estimates of herd exposure differed meaningfully.
The herd-level risk factors for WD that we identified were derived from case-control investigation of 12 WD affected Ohio dairy herds, and 24 unaffected control herds matched by geographical area. We concluded that recent herd exposure to BCV increased a herd’s risk for WD based on the herd prevalence of individuals within a herd demonstrating an IgG antibody seroresponse to BCV. This conclusion does not necessarily imply recent introduction of BCV onto the farm, but only re-exposure of each individual’s systemic immune system to BCV, leaving open the possibility that WD could be due to reactivation of persistent BCV infections on a farm, although this hypothesis was not tested in our studies.

Besides recent BCV exposure, other possible risk factors for WD were recent exposure to bovine viral diarrhea virus (BVDV); housing animals in tie-stall, or stanchion barns; or using the same equipment to handle feed and manure. The risk factors identified in this herd-level model of WD make biological sense when considered together. Both BCV and BVDV are respiratory and enteric pathogens, transmitted by aerosol inhalation and ingestion. Rapid spread of both pathogens could be facilitated orally by manure contamination of the feed, or by aerosol transmission when animals are tied head to head with limited air space in front of them in characteristically poorly ventilated buildings.

The impact of these risk factors individually on the WD experience of the population of Ohio dairies studied was substantial. Collectively this herd-level model of risk factors for WD explained 99% of the WD seen. However, we must acknowledge
that the strength of the associations measured are dependent upon the prevalence of the other components of sufficient cause to which these factors individually belong, and the prevalence of other sufficient causes that may be found in other populations. The use of stanchion barns as a risk factor compared to freestalls means little in populations where stanchion barns (or freestalls) do not exist.

8.4 Cow-level investigation of winter dysentery

Within WD affected herds some animals become clinically ill, while others do not, so there must also be cow-level risk factors that explain which animals get sick. In our study the pregnancy status of the individual was the single greatest predictor of the likelihood of being sick; pregnant individuals had 0.49 the odds of being a WD case compared to non-pregnant herdmates. We are the first to provide a statistical measure of association for pregnancy (or non-pregnancy) status and WD. This relationship has been noted but stated in different terms by others when they have suggested greater risk among recently fresh and high producing cows, which are nearly mutually exclusive of being pregnant. In multivariable models, neither days in milk at the time of the outbreak, as a categorical or continuous variable, nor measures of milk production were as informative as confirmed pregnancy for explaining the odds of being sick from WD. Other researchers have suggested that cows in early lactation, or high production may be immunologically compromised because of the stress of that phase of their production cycle. We argued, alternatively, that it may be that the immune modulation
that occurs during pregnancy, characterized by decreased humoral responses and increased non-specific immunity, that is protective of viral infection or disease.

As the BCV Ag ELISA value increased, the odds of being a sick individual increased. While it appeared that sick individuals shed more virus, this interpretation must be made with caution due to the limitations of the ELISA as previously discussed. For example it may also be that unaffected individuals also shed virus, but the virus was not detectable by ELISA due to antigen saturation by copro-antibodies.

There was a statistically significant interaction between the acute BCV IgG antibody titer and a BCV seroresponse; the odds of being sick increased with higher acute BCV antibody titers among those seroresponding to BCV, while the odds of being sick decreased with higher BCV antibody titers among those not seroresponding. This interaction was hypothesized and tested with the expectation that BCV exposed individuals with high acute BCV titers would be less likely to demonstrate a ≥4-fold rise in titer and so would appear to have lower odds of being sick. In fact, we found just the opposite to be true. This interaction, with high acute-titer seroresponders at greater risk of disease, could be evidence of antibody-dependent enhanced infectivity, or other immunopathology, or may be due to the dynamics of the assay or timing of sample collection; for example, since only a few days are needed to elicit a secondary immune response, these samples may have been from cows already sick for a few days. Further investigation will be necessary to clarify this finding.
The cow-level risk factors for WD were not clearly explained by this study. But it appears, from our cow-level model, that the reason that some cows get sick in WD-affected herds, somehow relates to the non-specific (effect of pregnancy) and BCV specific (acute serum BCV antibody titers, and BCV seroresponses) immunological profiles of the individuals. Previous researchers have suggested that younger stock are less susceptible to WD, and we demonstrated that pregnant animals appear to be less susceptible to WD. If the expression of WD in individuals mediated by an immunopathology, such as antibody-dependent enhanced infectivity, then we might speculate that younger stock and pregnant animals may be at reduced risk for BCV is because they may be less responsive immunologically.

8.5 What causes winter dysentery?

In addressing Campbell and Cookingham’s earlier speculations, we can say that the results of our study are consistent with one, or more, viral agents being associated with WD outbreaks. We have provided, for the first time, a statistical measure of association to link BCV with WD; and also suggest that recent herd exposure to BVDV may be causally associated with WD. Because both of these agents are enteric and respiratory pathogens, feed and aerosol contamination with these agents could explain the rapid transmission seen in WD outbreaks. The herd-level model also suggests mechanisms to facilitate the transmission of these agents by the type of housing (close head-to-head confinement) and the mechanics of the feeding process (manure contamination of the feed). The cow-level risk factors we found in the study
population suggest that the immunological profile of the individual, particularly as it might impact BCV infection, may have determined who got sick within WD affected herds.

We initiated these investigations of WD with the hypothesis that BCV was a causal agent of the disease. The evidence that BCV plays a causal role in WD outbreaks can be evaluated in the context of the causal properties defined by Susser: strength of association, time-order, and direction.\textsuperscript{11} We found a strong measure of association between BCV exposure and WD. The time-order of this association, that virus exposure precedes disease, has not been well documented. Our demonstration, and that of others, of rising antibody responses to BCV at the time of a WD outbreak suggests that this may be so, but does not refute the possibility that the disease leads to loss of mucosal immunity barriers and subsequent virus recrudescence and humoral antibody response. Additional evidence of time-order comes from experimental BCV infection studies; adult cows developed diarrhea and shed BCV in their feces after experimental exposure to BCV.\textsuperscript{\textsuperscript{11,12}} Prospective field studies, and further experimental infection studies will be necessary to strengthen the time-order argument. Direction is demonstrated after association and time order are established, by showing that changes in the cause produce changes in the effect.\textsuperscript{11} Our herd-level study hints that direction exists for the association of BCV and WD by demonstrating a biological gradient: that the risk for WD increases as the prevalence of BCV exposure increases within a herd.
However, a more definitive demonstration of direction for WD is needed and doing so will require prospective field investigation and experimental study designs.

Evaluation of BCV and WD in the context of Hill's causal criterion\(^\text{13}\) provides evidence for considering BCV as a causal agent of WD.\(^\text{8}\) Evidence of strength of association, temporality (time-order) and biological gradient for BCV and WD were discussed. Many researchers have found evidence for BCV infection of WD affected individuals and, along with our findings, demonstrate consistency. That BCV has been demonstrated to cause similar clinical signs and pathologic lesions in calves and adult cows, provides evidence of plausibility, analogy, and experiment. The herd-level and cow-level models we constructed for WD risk factors are coherent with current thinking on the pathogenesis of WD. Specificity of the association between BCV and WD has been refuted by previous evidence that BCV exposure causes other diseases, unless differences in neonatal calf- and WD-BCV are demonstrated (lacking specificity of effect). And our models, and the work of others,\(^{14-16}\) suggest that other agents and risk factors may also be causally associated with WD (lacking specificity of cause).

Many questions remain concerning the cause of WD. Future investigations will require improved measures of exposure, and increased specificity of disease classification. The role of BCV and the other risk factors identified in this study should continue to be studied by field investigation and experimentation, and the causal hypotheses further refined; these investigations should be conducted in varying
geographical regions. If evidence continues to accrue that BCV is frequently a risk factor for WD, then disease specificity would be enhanced if future investigations targeted "BCV-associated WD" or "non-BCV-associated WD" and risk factors were investigated, and further defined, for these more specific disease entities. Future measures of exposure at the cow-level will certainly need to include the assessment of systemic and mucosal immunological parameters.
8.6 Endnotes


b. See Chapters 1, 2, and 3.
8.7 References


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