Ecology of calf diarrhea in cow-calf operations

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Ecology of calf diarrhea in cow-calf operations

By

Yong-il Cho

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Veterinary Microbiology

Program of Study Committee:
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Iowa State University
Ames, Iowa

2012

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Dedication

To my family in physical and spiritual
Especially my wife Hyewan Lee and my son Ian Joonhyeop Cho

“Yahweh-jireh”
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ABSTRACT

Calf diarrhea is a commonly reported disease in young animal and still a major cause of productivity and economic loss to cattle producers. In the report of 2007 National Animal Health Monitoring System for U.S. dairy, a half of the deaths in unweaned calves were reported to be attributed to diarrhea. Multiple pathogens are known or postulated to cause or contribute to calf diarrhea. However, their significance and interaction in the disease has not been clearly addressed, not to mention uncertainty on the role of emerging viral pathogens such as bovine caliciviruses in calf diarrhea. It was of interest how diagnostic testing influences such an assessment. The long term goal of our study was to better understand the epidemiology, ecology and pathogenesis of well-known and potential bovine enteric pathogens in the field and developing better intervention strategies.

Specific aims of the current study were to: a) develop highly specific and sensitive diagnostic methods for simultaneous detection of major bovine enteric pathogens; b) determine the prevalence and molecular characteristics of bovine norovirus (BNoV) which has recently emerged as a potential enteric pathogen worldwide, in the US Midwest cattle farms; and c) characterize the epidemiology of historically well-known and emerging bovine enteric pathogens in calf diarrhea. A series of four studies were conducted to address these specific aims. In a separate study the prevalence of bovine enterovirus (BEV), bovine coronavirus (BCoV), bovine rotavirus group A (BRV-A) and coliform bacteria in pasture streams in Southern Iowa over a 3-year period was surveyed to assess their association with cattle grazing.
The objective of the first study was to develop a PCR panel (hereafter, bovine enteric panel) which can simultaneously detect multiple enteric pathogens with high sensitivity and specificity. The bovine enteric panel (BEP) was consisted of 2 multiplex real-time PCR assays for simultaneous detection of five major bovine enteric pathogens [i.e., BCoV (formally known as Betacoronavirus I), BRV-A, Salmonella spp. (Salmonella), Escherichia coli (E. coli) K99+, and Cryptosporidium parvum (C. parvum)]. The analytic sensitivity of the panel was 0.1 TCID$_{50}$ for BCoV and BRV-A, 5 and 0.5 CFU for E. coli K99$^+$ and Salmonella respectively, and 50 oocysts for C. parvum per reaction. Diagnostically, the panel was able to detect all five target pathogens directly from fecal samples and was more rapid and sensitive than conventional diagnostic tests (i.e., antigen-capturing ELISA, bacterial culture, direct microscopy with acid-fast staining).

The second study evaluated the diagnostic performance of a commercial “rapid” antigen detection kit named “Bovine Enterichek®” (hereafter, Enterichek) in comparison to BEP. The test agreement ($\kappa$ value) between Enterichek and BEP were 0.095 (BCoV), 0.521 (BRV-A), 0.823 (E. coli K99$^+$), and 0.840 (C. parvum). In comparison to BEP, the diagnostic sensitivity of Enterichek was 60.0%, 42.3%, 71.4% and 81.5%; and the diagnostic specificity was 51.4%, 100%, 100%, and 98.6% for BCoV, BRV-A, E. coli K99$^+$, and C. parvum, respectively. While Enterichek can be an animal-side or at-clinic rapid test tool in the field for detection of C. parvum or E. coli K99$^+$ in feces from calves at acute stage of clinical disease, BCoV positive and BRV-A negative results by the kit requires careful interpretation due to its relatively low specificity and sensitivity.
In the third study, the detection frequency and genetic relatedness of BNoV among bovine diarrhea cases in the US Midwest was assessed. Total 102 fecal samples were collected from clinically diarrheic animals originated in 82 different cattle farms in 8 states and tested by PCR-based assays. BNoV was detected in 53 samples (52%), suggesting endemic status in diarrheic bovine and emphasizing the need for further evaluation of its clinical significance. Among 38 BNoVs successfully sequenced for polymerase gene, 14 and 24 BNoVs were phylogenetically classified into GIII-1 and GIII-2, respectively. Interestingly GIII-1 BNoVs were identified at a much higher rate than expected based on previous reports in US. Clustering with ≥10% sequence divergence between clusters was observed within each genotype, justifying establishment of subtypes. Besides mutations, recombination among BNoVs appeared to occur frequently since the genotype of viruses was frequently switched when compared by capsid gene, raising the need for better classification criteria.

The fourth study was conducted in a case-control manner to: 1) survey the prevalence of 7 historically well-known and 4 emerging bovine enteric agents and 2) determine their association with calf diarrhea. Fecal samples were obtained from diarrheic (n=199) and healthy (n=249) calves and tested by multiple multiplex PCRs for the 11 enteric pathogens[BRV-A, BCoV, bovine viral diarrhea virus (BVDV), BEV, BNoV, Nebovirus, bovine torovirus (BToV), Salmonella, E. coli K99+, and Clostridium perfringens (C. perfringens) with β toxin gene, and C. parvum]. The association between the presence of pathogens individually or concurrently and diarrhea was analyzed by using a multivariate logistic regression model. More than a half of the fecal samples from the
diarrheic calves had multiple pathogens. Statistically, BRV-A, BCoV, BNoV, Nebovirus, *Salmonella, E. coli K99*+, and *C. parvum* were significantly associated calf diarrhea (*p*<0.05). Among them, BRV-A, Nebovirus, *Salmonella, E. coli K99*+, and *C. parvum* showed a stronger association with diarrhea, and BRV-A infection appeared to be responsible for ‘watery’ diarrhea. To our surprise none of the samples tested was positive for *C. perfringens* type C. Overall, viral etiology or co-infection of virus and *C. parvum* were the major contributor to calf diarrhea.

In testing 1274 water samples collected from 13 pasture streams in Southern Iowa during 2007-2009 grazing seasons for viral bovine biomarkers and coliform bacteria, BEV, BCoV and BRV were detected in 3.91%, 1.12% and 0.48% of the samples, respectively. There was a trend of BEV incidence difference between up- and down-stream sites, implying a dilution effect and/or loading of contaminant from the pasture. Total coliform bacteria counts did not correlate with BEV incidence as well as cattle presence or stocking density, indicating that other sources of fecal contamination may contribute to bacterial loading of pasture streams. Although the study results suggest that multiple factors affect the quality of pasture streams rather than solely cattle-originated contaminant, appropriate cattle grazing or pasture management practices should be considered to minimize bovine fecal contamination of pasture stream.

In conclusion, multiple pathogens were involved in calf diarrhea and frequently infected animals concurrently; hence, PCR-based panel testing for simultaneous detection of multiple pathogens may be a better way to assess their epidemiology and clinical significance in calf diarrhea. Historically known major bovine enteric pathogens, such as
BRV-A, BCoV, *Salmonella, E. coli* K99+ and *C. parvum*, were commonly and significantly associated with calf diarrhea, suggesting that lack of appropriate maternally derived herd immunity is still of concern for controlling calf diarrhea. Unexpectedly, bovine caliciviruses (i.e., BNoV and Nebovirus) were identified as significant bovine enteric pathogens. In particular BNoV was found to be widely distributed among diarrheic bovine in the Midwest USA with considerable genetic diversity. These observations raise the need to pay attention to these emerging pathogens for better control of bovine enteric diseases. Transmission of enteric pathogens through pasture streams is unlikely although bovine feces can be a source for microbial contamination of stream water, which could be minimized by better grazing management.
CHAPTER 1. GENERAL INTRODUCTION

THESIS ORGANIZATION

This dissertation consists of six chapters. Chapter 1 is the general introduction providing a broad overview of bovine enteric pathogens, diagnostic methods for those pathogens, and prevention and control of calf diarrhea. Chapter 2, “Development of a panel of multiplex real-time PCR assays for simultaneous detection of major agents causing calf diarrhea in feces” is a research article that has been published in the Journal of Veterinary Diagnostic Investigation. Chapter 3, “Evaluation of a commercial rapid test kit for detecting bovine enteric pathogens in feces” has also been published in the Journal of Veterinary Diagnostic Investigation. Chapter 4, “Detection and molecular characterization of bovine norovirus among bovine diarrhea cases in the Midwest USA” has been prepared to be submitted to the Journal of Clinical Microbiology. Chapter 5, “A case-control study of microbiological etiology associated with calf diarrhea” is in preparation for submission to the Veterinary Microbiology. In addition, a paper in the appendix, “Longitudinal study of bovine enteric virus incidence in Midwestern pasture streams for 2007 to 2009 grazing seasons”, has been prepared for submission to the Applied and Environmental Microbiology. References, tables, and figures for each research manuscript follow the discussion section of each. The last chapter contains the general conclusions of the dissertation research.
LITERATURE REVIEW

Introduction

Calf diarrhea is a commonly reported disease by and a major cause of economic loss to cattle producers. The 2007 National Animal Health Monitoring System (NAHMS) for U.S. dairy (164) reported that 50% of the mortality in unweaned calves were due to diarrhea and most occurred in calves at less than 1 month of age. Diarrhea is attributed to both infectious and non-infectious factors (9, 73, 80). Multiple enteric pathogens (e.g., viruses, bacteria and protozoa) are involved in the disease and co-infections are frequent in diarrheic calves, as well as single primary pathogen (170). The prevalence of pathogens and disease incidences are also influenced by geographical locations of farms, farm management and herd size.

Infectious factors

Numerous infectious agents have been implicated in calf diarrhea. Many of the enteric pathogens are well known to bovine practitioners and cattle producers because these primary pathogens have been involved in calf diarrhea for several decades. Ten different enteric pathogens are recognized as either major (bovine rotavirus, bovine coronavirus, bovine viral diarrhea virus, Salmonella spp, Escherichia coli, Clostridium perfringens, Cryptosporidium parvum) or emerging pathogens (bovine caliciviruses, bovine torovirus).
Viruses

Bovine rotavirus (BRV) is one of the primary etiologies of calf diarrhea. The virus belongs to the genus *Rotavirus* within the family *Reoviridae*. Rotavirus is a non-enveloped virion possessing 11 double-stranded RNA segments (16-21kbp) (44). There are 7 serogroups (A through G) among rotaviruses based on antigenic and genetic similarity of the intermediate capsid protein (VP6) (158). Group A rotavirus is the major cause of rotaviral infection in domestic animals. Most of BRVs (95%) belong to group A, although groups B and C rotaviruses have also been identified in the field cases (52, 93, 162).

Group A rotaviruses can be further classified into P or G types based on the genetic and antigenic similarity of VP4 (protease sensitive protein) and VP7 (glycoprotein) which constitute the outer capsid of the virion and induce anti-viral neutralizing antibody (30). The 16 G types and 27 P types have been reported in domestic animals. Bovine rotaviruses belong to G1, G6, G8, or G10 types (56, 81, 98, 138). G6 and G10 type are reported to be the most prevalent in cattle (30, 98).

While VP4, VP6 and VP7 play a major role in maintaining viral structure, virus attachment and antigenicity, nonstructural glycoprotein 4 (NSP4) has a special role as viral enterotoxin and interferes with cellular homeostasis by elevating calcium ion influx into cytoplasm (5). Such an alteration accounts for the drastic change in movement of nutrients and water across the intestinal epithelium and is more important for the viral pathogenesis than histopathological lesions.

Bovine rotavirus usually causes diarrhea in calves at 1 to 2 weeks of age (18). The milk uptaken by calf can provides a good environment for rotavirus survival at a wide range of gastrointestinal pH levels and the virus infecting the intestinal epithelial cell (31).
This may explain that unweaned calves are more susceptible to calf diarrhea. The virus has a very short incubation period (12-24 hours) (158) and induces peracute diarrhea in affected calves. The virus replicates in the cytoplasm of epithelial cells of small intestinal villi (68). Thus, destruction of mature enterocytes in the villi, activation of the enteric nervous system by vasoactive components from the damaged cells and secretion of a viral enterotoxin (e.g., NSP4) account for the maldigestive/malabsorptive diarrhea by rotavirus infection. Evidence for interspecies transmission and for genetic reassortment between human and animal rotaviruses (e.g., swine, bovine, feline and canine) has raised the zoonotic concern of rotaviruses (97).

**Bovine Coronavirus (BCoV)** is an enveloped virus with positive-sense, single-stranded RNA genome (27-32kb) and is a member (*Betacoronavirus 1*) of the genus *Betacoronavirus* which was formerly classified as group 2a coronaviruses (29, 62). Virus infection can present 3 distinct clinical syndromes in cattle: a) calf diarrhea in calves at 1 to 2 weeks of age; b) winter dysentery with hemorrhagic diarrhea in adults; and c) respiratory disease in both young and adult cattle including the bovine respiratory disease complex (19, 91).

The spike (S) protein of the virus plays an important role in virus entry and pathogenesis besides its antigenic importance for neutralizing antibody (89). The S protein consists of two subunits (S1 and S2) and has a crucial role in virus-host interaction. While S1 subunit functions in binding of the virus to host cell receptor, S2 subunit functions in fusion of viral envelope to host cellular membranes (145, 179).
The infection begins in the small intestine and usually spreads through the entire small intestine and colon. Initially, the S protein and hemagglutinin-esterase (HE) protein of the virus attach and fuse onto intestinal epithelial cells. (128, 147). The virus replicates in enterocytes and progeny viruses are released through normal secretory mechanism and cell lysis. The mature villous epithelial cells are the primary target for the virus, but crypt enterocytes are also affected (126). The clinical signs in affected animals often have a longer duration due to the damage done to crypt enterocytes by the virus.

**Bovine viral diarrhea virus (BVDV)** is an enveloped, positive-sense, single-stranded RNA virus (12.3kb) and is a member of the genus *Pestivirus* in the family *Flaviviridae* (47). There are 3 species present within the genus, BVDV, border disease virus, and classical swine fever virus (142). Bovine viral diarrhea viruses can be classified into 2 types (BVDV1 and BVDV2) based on the sequence similarity of 5’ untranslated region (UTR) in the viral genome. Each type can be further divided into 2 biotypes (cytopathic and noncytopathic) based on their ability to cause lytic cytopathic effect in cell culture. Noncytopathic strains of BVDV are responsible for persistent infection of the virus in cattle (60). To date 15 (BVDV1a to BVDV 1o) and 2 (BVDV2a and BVDV2b) subgenotypes have been recognized within BVDV1 and BVDV2, respectively (47, 75). BVDV1a, BVDV1b, and BVDV2a are most prevalent subtypes in US cattle populations (51).

The clinical symptoms of BVDV infection vary from subclinical to fatal disease depending upon host immune statue, pregnancy status and gestation period, and presence and absence of co-infection with other pathogens. Most infected animals show mild
clinical signs such as mild fever, leukopenia, anorexia and decreased milk production. The acute BVD is characterized by diarrhea, pyrexia, depression, anorexia, decreased milk production, oral ulcerations, hemorrhagic syndrome and lymphopenia/leucopenia leading to immunosuppression (3, 129, 172). Immunosuppressed cattle become susceptible to other diseases due to the concurrent infection (e.g., bovine respiratory disease complex). Although most of immunocompetent animals eventually clear the virus and recover from the disease, some of infected animals occasionally become transiently infected with BVDV(64).

Pregnant cows and heifer deliver persistently infected (PI) calves if they are exposed to a non-cytopathic BVDV during 45-125 days of gestation as fetus is not immunocompetent (114). Most PI calves are born weak and susceptible to other pathogens and have poor growth performance. The PI animals develop fatal “Mucosal Disease” when exposed to a either exogenous or endogenous cytopathic BVDV (12). Mucosal disease is characterized clinically by mucosal ulceration, vesicle formation, erosions, diarrhea and death (64).

BVDV can be involved in calf diarrhea in two major ways: 1) Persistently infected with primary damage to enterocytes and immunocompromise to coinfections; or 2) Transient infection with replication and lesions in crypt enterocytes contributing to diarrhea.

**Bovine torovirus (BToV)** is an enveloped, positive-stranded, RNA virus (25-30kb) and is classified into the genus *Torovirus* in the family of *Coronaviridae*, order *Nidovirales* (83, 154) along with equine torovirus, porcine torovirus and human torovirus.
Toroviruses have been reported as an infectious gastrointestinal agent in cattle (67) and have been a cause of enteric infections in piglets and humans (84, 92). Fecal shedding of BToVs from diarrheic calves have been reported around the world: United States (2003, 2002, 1983 and 1982), Canada (1998), Costa Rica (1998), South Korea (2008), Netherlands (1991), Germany (1992), Hungary (2002), Austria (2006), Japan (2007), and South Africa (1993) (34, 61, 66, 82, 127, 131, 168). Morphological similarity and antigenic cross reactivity between human and bovine toroviruses has raised a concern regarding potential zoonotic nature of BToV (67).

Bovine torovirus can produce mild to moderate diarrhea in calves under both field and experimental conditions (13). Upon oral or nasal inoculation of the virus, the virus infects epithelial cells in the middle and lower parts of intestinal villi extending into the crypt epithelium and induces cell death and epithelial desquamation in the small intestine, together with necrosis in the large intestine (37, 135). The damage to the villous and cryptic enterocytes thus induces a malabsorptive/maldigestive diarrhea. Thirty to 50% of lesions caused by the virus are present in the upper small intestine, which may explain mild to moderate diarrhea in the affected animals (176). Similar to BCoV, BToV antigen and viral RNA have been detected in nasal secretions, but its role in respiratory disease remains to be further studied (65).

**Bovine norovirus (BNoV)** is a non-enveloped, single-stranded positive-sense RNA virus (7.4-8.3kb) belonging to the genus *Norovirus* in the family *Caliciviridae* (21, 90). Five genogroups (GI through GV) have been identified based on the sequence similarity of ORFs 2 (VP1: major capsid protein) and 3 (VP2: minor capsid protein) due to high genetic
diversity among noroviruses (NoVs) (180). Bovine noroviruses belong to GIII that has
two prototype strains, Jena (genotype 1; GIII-1) and Newbury 2 (genotype 2; GIII-2)
viruses, and is phylogenetically distinct from human (GI, GII and GIV), porcine (GII-11,
GII-18 and GII-19) and murine (GV) NoVs (100, 119, 149). The possibility of
interspecies transmission of NoV was shown in a study demonstrating infection of
gnotobiotic pigs by a human NoV strain, raising a concern for its zoonotic potential
worldwide (17, 100).

Numerous studies have been conducted to survey BNoV infection in cattle and to
molecularly characterize the viruses in comparison to human NoVs. The reported
frequency of BNoV detection as measured by molecular methods widely varied among
different countries, ranging from 7.5% to 49.6% (32, 77, 79, 101, 120, 123, 140, 151, 165,
178). All identified BNoVs to date have been phylogenetically distinct from human NoVs,
suggesting that zoonotic potential of BNoV is unlikely.

Noroviruses are a major cause of non-bacterial acute and sporadic gastroenteritis in
humans (96). Noroviruses have also been reported to cause gastroenteric disease in
animals such as cattle, pigs, dogs and mink (149). Recently, an experimental challenge
study with the Jena strain of BNoV was conducted on newborn calves via oral route (121).
The investigators demonstrated that the virus infected epithelial cells of small intestine and
caused villous atrophy (jejunum and ileum) leading to diarrhea with shedding but did not
induce seroconversion detectable by an ELISA. Detection of BNoV in feces from
clinically healthy cattle has also been reported by a few investigators (77, 107, 140).
Neboviruses belong to the newly established genus *Nebovirus* in the family *Caliciviridae* (15). The viral genome is approximately 7.4kb in length and contains two open reading frames (ORF): ORF1 (polyprotein producing nonstructural proteins and capsid protein) and ORF2 (small basic proteins with unknown function) (118, 150). Newbury agent-1 and the Nebraska-like bovine calicivirus are two distinct genotypes which were reported in association with calf diarrhea cases in UK (1978) and Nebraska USA (1980), respectively (118, 150, 175). Since then, the presence of Nebovirus has been reported from other countries, such as France (2011), Italy (2011) and Korea (2008) (33, 79, 124). The reported prevalence of Neboviruses in the studied diarrheic calves ranges from 7% to 28.0%, depending upon geographic location (33, 79, 118, 124). There is no evidence of zoonotic transmission. Genetic diversity has been reported to exist among Neboviruses with identifying a novel genotype (79). Similar to BNoV, lesions by Nebovirus are observed mainly in the jejunum and ileum with villi atrophy, loss of villi enterocyte and crypt hyperplasia when gnotobiotic calve were challenged with the virus (58, 150).

**Bacteria**

*Salmonella enterica* colonizes the gastrointestinal tract of a wide range of hosts (177). *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) and serovar Dublin (*S. dublin*) are most common etiology of salmonellosis in cattle (71, 155). *S. typhimurium* is the most common serotype affecting calves in the United States (144).

Salmonella infection has a wide range of clinical manifestation from asymptomatic to clinical salmonellosis. Acute diarrheal disease is most common with *S. typhimurium* and
systemic disease with *S. dublin* in cattle. Infected cattle can serve as source of zoonosis through food-born or direct contact routes (103).

The basic virulence mechanism of *Salmonella* includes the ability to invade the intestinal mucosa, to multiply in lymphoid tissues, and to evade host defense systems, leading to systemic disease. For the pathogenesis of *Salmonella*, the organism should be capable of invading intestinal epithelial cells, surviving within macrophages and causing enteropathogenicity (27, 161, 169). *Salmonella* colonizes in M-cell, enterocytes and tonsilar tissue (139). Lymphoid tissue (e.g., tonsilar tissue) infection, *Salmonella* easily spreads throughout the whole body by invading mononuclear cell and phagocytes (69). *Salmonella* pathogenicity island 1 (SPI-1) and SPI-5 are known to be involved in the type III secretion system and are mainly responsible for *Salmonella* induced diarrhea in calves (22, 161, 174). SPI-2 is involved in the second type III secretion system and is responsible for intracellular survival of the organism (116).

The clinical presentation of salmonellosis is characterized by watery and mucoid diarrhea with the presence of fibrin and blood (48). Even though *Salmonella* can cause diarrhea in both adult cattle and calves, infection is much more common and often causes severe symptoms in 10-day to 3-month old calves (48).

*Escherichia coli* (*E. coli*) can be classified into 6 pathogroups based on their virulence scheme: enterotoxigenic *E. coli* (ETEC); shiga toxin-producing *E. coli*; enteropathogenic *E. coli*; enteroinvasive *E. coli*; enteroaggressive *E. coli*; and enterohaemorrhagic *E. coli* (78, 111). Among these pathogroups, the most common cause of neonatal diarrhea is ETEC stains that are producing the K99 (F5) adhesion antigen (commonly referred to as *E. coli*
K99\(^+\)) and also the heat-stable enterotoxin (111). It should be noted that other pathogroups of \textit{E. coli}, which are usually implicated by histopathology, can be missed if the diagnostic focuses only on \textit{E. coli} K99\(^+\).

Neonatal calves are most susceptible to ETEC infection during first 4 days after birth and develop “watery” diarrhea if infected (49). Following ingestion, ETEC infects the gut epithelium and multiplies in enterocytes in intestinal villi. The distal portion of small intestine is the most favorable environment of ETEC colonization due to the low pH (less than 6.5). The bacteria expresses the K99 antigen for the attachment (50). As colonized on the gut epithelium, heat stable toxin is induced by ETEC and causes the secretory diarrhea.

\textit{Clostridium perfringens} (\textit{C. perfringens}) is a gram-positive, spore-forming anaerobic bacterium causing a wide range of diseases in mammals and birds (102, 157, 166). It can be subdivided into five toxin types (A, B, C, D and E) based on the production of four major toxins; alpha, beta, epsilon and iota (132). Type A strains produce alpha (\(\alpha\)) toxin only; type B stains produce \(\alpha\), beta (\(\beta\)), and epsilon (\(\varepsilon\)) toxins; type C type strains produce \(\alpha\) and \(\beta\) toxins; type D strains produce \(\alpha\) and \(\varepsilon\) toxins; and type E strains produce \(\alpha\) and \(\iota\) (iota) toxins. Among these types, type C has been frequently reported in conjunction with calf diarrhea (143) but not as frequently as some other enteric pathogens such as BRV, BCoV, \textit{E. coli}, \textit{Salmonella} and \textit{Cryptosporidium parvum}.

The \(\alpha\) toxin is the main lethal toxin, and functions in cell lysis through hydrolysis of membrane phospholipids. The \(\beta\) toxin is highly trypsin-sensitive and induces mucosal necrosis. The \(\varepsilon\) toxin causes lethal enterotoxemia in domestic animal, and the \(\iota\) toxin is
responsible for dermonecrosis due to its high vascular permeability (72, 132, 156, 157). Enterotoxin causes diarrhea and intestinal cramping due to its act on epithelial tight junction protein. Beta-2 toxin, which is produced from all types of *C. perfringens*, has been recently reported and postulated to have a synergetic function with enterotoxin (57).

Most domestic animals are susceptible to all types of *C. perfringens* due to the ubiquitous nature of the bacterium in the environment. Newborn calves which have a low level of proteolytic enzymes (e.g., trypsin) in gastrointestinal track can be easily infected by *C. perfringens* type C as β toxin is recognized as the main virulence factor responsible for clinical signs seen in affected animals. Intestinal lesions in such affected animals are characterized by diffuse or multifocal hemorrhagic necrotizing enteritis and bloody fluid distension (7).

Protozoa

*Cryptosporidium parvum* (*C. parvum*) is a protozoan parasite that is frequently associated with gastrointestinal tract disease in humans and neonatal cattle. Infection with *C. parvum* in calves can be from asymptomatic to severe diarrhea with dehydration (41, 42). There are approximately 24 species of *cryptosporidium* (39, 43). Cattle are commonly infected by *C. parvum*, *C. bovis*, *C. ryanae* and *C. andersoni*. *Cryptosporidium parvum* is considered as primary cause of calf diarrhea and is a potential zoonosis (16, 42).

Once *C. parvum* is ingested, the oocyst excystation releases sporozoites which penetrate enterocytes. The excysted parasites undergo asexual (type I meront) and sexual reproduction (type II meront), producing macrogametocytes and microgametocytes. Upon fertilization of the macrogametocytes by microgametes, zygotes are developed which
sporulates (*sporogony*) generating *thin-walled* oocysts involved in autoinfection. Then *thick-walled* oocysts pass out from the host. The oocysts can survive more than a month in the environment under favorable conditions (e.g., high temperature, moisture and low UV radiation) and are resistant to most disinfectants (40). The environment contaminated with oocysts can be an immediately source of infection in both animals and humans.

The invasion of *C. parvum* in enterocytes induces changes in cytoskeletons of intestine, such as loss of microvilli and shortening of columnar epithelial cells, leading to severe villous atrophy in infected animal (63). Damage to the intestinal epithelium causes prolonged malnutrition and reduced growth rate in affected calves due to malabsorption and fermentation of undigested milk in the intestinal lumen, resulting in considerable economic losses in cow-calf production (113).

**Diagnosis of calf enteric pathogens**

Diarrhea can be fatal to neonatal calves due to anorexia, dehydration and ataxia (11). Progression of diarrhea can be rapid; hence, accurate and rapid diagnosis is critical for not only confirming the cause but also aiding implementing appropriate interventions.

Proper specimen collection and delivery to a diagnostic lab is commonly neglected but significantly impacts the diagnostic outcome. Antemortem samples for diagnostic testing should minimally include feces from acutely diarrheic animals prior to therapy, with optional blood samples. Necropsy specimens from freshly dead, moribund, or euthanatized calves are of great value for diagnosis of severe outbreaks. Fresh and formalin-fixed gastrointestinal tissues (abomasum, small intestine, colon) including regional lymph nodes and liver along with colon content should be collected (23). Fresh
fecal samples should be collected directly from diarrheic animal into a specimen container through either rectal swabs or rectal stimulation without environment contamination (soil, urine, or other feces). Once collected, the sample should be stored in a transporting medium or special stool container under refrigerated conditions to maintain viability of pathogens and integrity of sample (i.e., reducing overgrowth of undesired bacteria, preventing nucleic acid degradation) (76). Samples for anaerobic bacteria (e.g., *C. perfringens*) should be kept in an oxygen-free transport medium during shipping if possible.

Laboratory test methods for enteric pathogens have typically included pathogen isolation and identification and histopathology as the gold standard for agent and disease confirmation (137). However, many of enteric pathogens are difficult to isolate from gastrointestinal environment conditions (36, 59). Direct visualization (e.g. microscopy, electron microscopy) of pathogens in feces or intestinal contents or detection of antigens (e.g., antigen-capturing ELISA) or nucleic acids (e.g., PCR) of pathogens in specimens (feces, content and intestine) have been widely accepted alternative methods. Most of veterinary diagnostic laboratories use a variety of tests concurrently when to test the samples for enteric pathogens. Characteristics, advantages and disadvantages of laboratory methods which are commonly used for enteric pathogens are briefly described below.

**Virus isolation test** is still considered as ‘gold standard’ for detecting the presence of a viral pathogens in specimens (137), although there have been new test methods such as enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR). Cell culture techniques are commonly used for virus isolation for diagnostic purpose, as well as
virus propagation for vaccine production or further virus characterization such as antigenic variation or gene sequencing (141). There are several cell lines (e.g., Madin Darby bovine kidney cells, human rectal tumor cells and African rhesus monkey kidney cell MA104) used for certain viruses because the susceptibility of different cell lines to viruses varies (1, 2). The viability of target virus in a specimen is critical for the success of virus isolation (146). Specimens should be kept at a low temperature and in a transport medium during shipping to a diagnostic laboratory and delivered to the lab as soon as possible after collection. Virus isolation test is the confirmatory test; however, it takes a time for preparing cells and propagating virus (i.e., slow turnaround of results) and is laborious and expensive as compared to ELISA or PCR methods.

**Electron microscopy (EM)** is commonly used for virus detection and identification based on morphological characteristics. There are two types of EM methods: direct EM and immunoelectron microscopy (IEM) (13). Two different staining techniques (positive and negative staining) are used to visualize the presence of target. In the direct EM, virus particles in a fluid sample matrix are applied directly to a solid support and then are visualized by EM after a contrast stain is applied. It is commonly referred to as “negative straining EM”, whereas positive staining is generally used in a thin-section EM on fixed tissues. In comparison, IEM has a higher sensitivity and specificity than direct EM as a specimen is incubated with antibody specific for the target virus in order to agglutinate the virus before staining.

The visualization of viruses, particularly non-cultivatable ones, is a major advantage of EM with rapid turnaround. Most of bovine enteric viruses, such as BNoV,
Nebovirus, BRV, BToV and BCoV, are difficult to isolate or propagate in cell culture, but these viruses can be differentiated by their morphology under an electron microscope (34). The EM test requires a large number of virus particles (e.g., approximately $10^6$ virus particles per $\text{mL}$) present in specimen for virus detection (i.e., low sensitivity) and cannot test multiple samples concurrently (45). The cost of electron microscopes and requirement of skilled laboratory personnel is still a challenge for the EM test being used as routine diagnostic test.

**Antigen capturing enzyme-linked immunosorbent assay (Ag-ELISA)** is an assay for rapidly detecting a pathogen in a clinical specimen based on antibody (e.g., monoclonal antibody) recognition of the target antigen (88). It has antibody attached to a solid surface which can be a glass, plastic material or membrane filter. This antibody captures the target antigen if present in the sample. Then there will be a cascade of colorimetric reactions to verify capturing of the antigen and visualize the antigen-antibody reaction. Antigen can be quantitatively estimated as optical density (OD) measured by a spectrometry positively correlates with the amount of antigen.

Antigen-capturing ELISA technique has been utilized in many areas. Several platforms are used: tube method, microtiter plate method and membrane-bound method (45). While microtiter plate method has been commonly employed in a diagnostic laboratory setting, membrane-bound method is the most common platform for at-clinic or patient-side test. There are commercial Ag-ELISA kits for BRV-A and BCoV in fecal samples (94, 125). The antigen-capturing ELISA is well known for its rapid turnaround, high-throughput testing, plug-in-and-play capability, and portability (45). Its analytic
sensitivity tends to be lower than that of isolation or nucleic-acid based assays (20). In some situations, the expense of the commercial kit may be cost-prohibitive, particularly for veterinary medicine.

**Fecal flotation and direct microscopy** are commonly used for parasite eggs or oocysts. The principal of fecal flotation is simply based on the density difference between flotation solution ($\geq 1.24$) and oocysts (1.05-1.24) (6). A centrifugation step is commonly included in testing procedure to increase the detection sensitivity as the centrifugation concentrates the target for easy viewing under a microscope. Direct microscopy can also apply to fecal smears without centrifugation.

Oocysts in clinical specimens may be difficult to visualize without special staining. *C. parvum*’s oocysts are reported to stain positive with acid-fast staining (109). Modified acid-fast stains are applied to fecal smears to detect these organisms. Different from the Ziehl-Neelsen modified acid-fast stain, the modified Kinyoun acid-fast stain uses more concentrated fuchsin dye and lipid solvent and does not require heating reagents used for staining (95, 160). In brief, 1 to 2 drops of feces is smeared on a clean glass slide and allowed to be air dried. The glass slide is fixed with absolute methanol and subsequently stained with carbol fuchsin and 1% sulfuric acid. The slide is then counterstained with methylene blue or brilliant green and examined under a light microscope with oil immersion. The red or purple colored oocysts of 4 to 6 µm in diameter in case for *C. parvum* should be observed against a blue or green background. This modified acid-fast staining method is widely used to detect *C. parvum* in feces. Its sensitivity is low because
the method requires the presence of approximately 500,000 oocysts per 1 gram of feces to confirm \textit{C. parvum} oocyst (4).

\textbf{Fecal bacteria culture} is a commonly used laboratory test to isolate and identify bacterial pathogens from fecal samples. \textit{Salmonella} spp., \textit{E. coli} K99\(^{+}\) and \textit{C. perfringens} are primary bovine enteric bacterial pathogens (46, 73). In order to prevent any cross-contamination, feces should be collected directly from diarrheic calves through the either rectal swabs or rectal stimulation. Once collected, fecal samples should be stored in a transport medium or special stool container in a cooler or on ice for submission to a diagnostic lab in order to minimize loss of viability. For anaerobic bacteria like \textit{C. perfringens}, fecal samples must be immediately kept in a pre-reduced (i.e., oxygen free) transport medium if available.

Blood agar plate, MacConkey agar plate, MacConkey agar with sorbitol, Hektoen enteric (HE) and XLD (xylose lysine desoxycholate) plate are used for bacterial culture (26, 115). Several kinds of enriched and selective media, such as brain heart infusion (BHI) broth (a high nutritious medium for general-purpose bacterial culture) and tetrathionate broth (for \textit{Salmonella} spp.), are employed for growth and identification of certain bacterial pathogens. Blood agar plate is most commonly used because most of bacteria can be grown on this plate. MacConkey agar is selectively used to culture gram-negative bacilli which are commonly present in gastrointestinal track and is able to differentiate the bacteria fermenting lactose. Sorbitol-MacConkey agar can differentiate the nonpathogenic \textit{E. coli} from \textit{E. coli} O157:H7 which cannot ferment sorbitol (38). \textit{Salmonella} spp. are commonly cultured out of feces by using SS agar, bismuth sulfite agar, HE medium,
brilliant green agar and XLD agar (167). For *C. perfringens*, thioglycolate broth growth medium is commonly used. Culture usually takes two day at 36°C in anaerobic condition(46). The shape of colonial growth (e.g., shape, surface and elevation level of colony on agar plates), physical characteristics (e.g., aerobe, anaerobe or microaerophile), microscopic features (e.g., rods, cocci or coccobacilli) and biochemical tests (e.g., such as test to confirm fermentation, gelatin or urea utilization, indole, oxidase or catalase production, etc.) are then used to characterize and identify the isolated bacteria. Slow turnaround of the result is a disadvantage of bacterial culture test since growth and identification can take 24-72 hours, although the turnaround can vary depending on culture methods and diagnostic instruments. In some cases, further immunological testing (e.g., latex agglutination test) is required for identification of bacteria (e.g., *E. coli K99*).

**Latex agglutination test (LAT)** is in principle similar to ELISA test (136). Antigen or antibody is coated on the surface of latex particles, which captures antibody and the target antigen, respectively. The test has been applied to detection of a wide range of targets, such as bacteria, virus, hormones, drugs and serum protein (122).

Latex particles are made of synthetic rubber and emulsified as billions of micelles of the same size of a desired diameter. Usually the size of particles ranges between 0.05 to 2 µm in diameter, and the presence of sulfate ions provides an inherent negative surface charge to the particles (130). This prepared latex particle can be further functionalized by special processing, such as amidation, amination, carboxylation, hydroxylation or magnetization, to increase their binding stability and analyte attachment depending upon the purpose of test (130).
Related to calf diarrhea, LAT has been frequently used to identify *E. coli* K99+ (20). For testing, fecal samples are collected from diarrheic calves and sent to a diagnostic lab for testing. Once *E. coli* is isolated, a bacterial suspension is mixed with latex beads specifically coated with anti-*E. coli* K99+ antibody and incubated according to the manufacturer instructions. The agglutination of the latex beads can be clearly visualized in a shape of clumping if K99 antigen is present in the isolated *E. coli*.

The latex agglutination test is frequently employed in diagnostic labs because it can be a semi-quantified test and is relatively cheap with rapid turnaround (54). Caution should be taken in interpreting marginal results as false positive/negative results frequently occur due to non-specific binding or interference (136).

**Polymerase chain reaction (PCR)** is now a commonly used test method for detecting enteric pathogens. PCR is a thermocyclic enzymatic amplification of specific sequence of the target pathogen using a pair of oligonucleotide primers that hybridize on each DNA/cDNA strand of interest region in the genomic sequence. For PCR testing, the genomic material of the target pathogen is first obtained by extraction procedure. After the extract that contains templates (i.e., genomic material of the target pathogen) is mixed with a heat stable DNA polymerase (e.g., Taq DNA polymerase), dNTPs, primers and PCR buffer reagents, the amplification reaction usually runs on the mixture for 25 to 40 cycles in an automated thermal cycler (35). Each cycle includes denaturing of double-stranded DNA, annealing of primers on each DNA strand and polymerization of a new strand. After completion of the reaction, the PCR products can be visualized on an agarose or acrylamide gel by electrophoresis technique and special staining with ethidium bromide.
which is bound to double-stranded DNA (unless a real-time PCR described below is used). Amplification of the target sequence is determined based on molecular size and/or sequencing of the PCR product.

PCR testing is especially useful to detecting viruses which are difficult to isolate in cell culture or for bacteria which take a long time to grow on routine culture or enriched growth media (36, 59). There are numerous commercial PCR reagents available which provide convenience, high sensitivity and rapid results. PCR testing requires a trained and experienced technician with skill. Inadvertent contamination during processing can be a source of false positive results due to its high sensitivity. Viruses with high mutation rate, often RNA viruses (e.g., rotavirus and calicivirus), need to be continuously monitored for their sequence changes in the target gene (74); otherwise, PCR tests can give negative results due to primer incompatibility.

**Real-time PCR** is a PCR method which, when compared to gel-based conventional PCR, amplifies the target sequence and also quantifies the amount of the target with higher sensitivity (173). There are two types of real-time PCRs commonly used for diagnostic purposes: TaqMan® and SYBR® Green real-time PCR. TaqMan® real-time PCR has an oligonucleotide probe labeled with two types of fluorophores (i.e., report dye and quencher dye) in addition to a primer pair (36). The reporter dye is located on the 5’ end of the probe and the quencher at the 3’ end. After denaturation of the DNA template during PCR reaction, primers and probe bind to each strand of the template. Extended primer removes TaqMan® probe from the template DNA, resulting that the reporter dye is separated from the quencher dye. The emission from the reporter dye (e.g., fluorescence energy) can be
detected by spectrophotometric mechanism of a real-time PCR instrument. All processing steps of real-time PCR are conducted in a closed tube system; hence, the opportunity of contamination can be minimized. The assay provides high specificity because of signal detection of probe according to the primer sequence extension.

The principle of SYBR® Green real-time PCR is based on binding of SYBR® Green dye to double-stranded DNA which will emit the light when excited. SYBR Green assays are cheaper than TaqMan® real-time PCR assays. However, the dye binds to any of double-stranded DNA. Therefore, SYBR® Green real-time PCR requires a melting-curve analysis to determine whether the amplification curve is coming from the intended target or others such as primer dimmers or non-specific amplicon (70).

There are several kinds of reporting dyes used with probe-based real-time PCR assays based on their wave length of fluorescence energy, which makes multiplexing possible through combination of different reporting dyes. Theoretically, a multiplex real-time PCR can simultaneously detect up to 4 different targets in the same sample in a differential manner (20). However, there is a limit in the size of the PCR product, which is usually less than 200 bp, in order to keep the stable sensitivity (171), requiring careful design of primers and probes when a multiplex real-time PCR assay is desired. “Cross-talking” between different dyes due to a close proximity in wave length of fluorescence energy is another factor to take into consideration for multiplexing.
Prevention and control of calf diarrhea

Calf diarrhea is a multifactorial disease (9, 28, 73, 80, 163). The factors involved in the occurrence of calf diarrhea can be summarized as: a) peripartum calving management, b) calf immunity; and c) environment stress and contamination. The characteristic of major or emerging bovine enteric pathogens are described above. There is not much of difference in the pattern of disease and prevention of calf diarrhea according to each etiology. Knowing of causal pathogen is important for characterizing the current problem of the index farm and developing further intervention for the disease. Nowadays, the disease control and prevention in production animals faces two aspects: a) animal welfare at public or consumer’s point of view; and b) increased productivity at livestock producer’s point of view.

Peripartum calving management

Cow nutrition is closely associated with weak labor, amount of milk production, dystocia and calf growth (reference). Inadequate feed intake or macro- or micro-nutrient deficiencies during last trimester increases calve morbidity and mortality rate because the most fetal growth occurs during last two months of gestation (24, 105, 106). The quality and quantity of colostrum is associated with body condition score (BCS). A BCS near 5 (scale 1-10) is acceptable for multiparous cows and 6 in primiparous cows at calving (85). Recently, cow nutrition has shown an impact on the transition of the calf into adult life as well as fetus growth and development (53, 55). Calves born to underfed cows had poor growth performance, low productivity and higher susceptibility to disease. In another study, heifer calves born to cows fed on supplemental protein during the last trimester were
shown to have greater pregnant performance later in her life compared to the control group (99).

**Dystocia** is closely related with poor calf performance, increasing calf’s susceptibility to environmental pathogens which frequently cause calf diarrhea (86). Calves that experience dystocia may have physical injuries, such as congestion and swelling of head and tongue (152), which can reduce the amount of colostrum uptake from dam. The absorption rate of colostrum-derived immunoglobulin is lower in those calves than healthy calves (117). Consequently, those calves cannot get appropriate passive immunity from dams because of inadequate colostrum uptake within early calf life (i.e., 6-12 hours after birth).

The major causes of dystocia are related with large calf size and small pelvic size of dam (86). Large calves are more likely to have improper position and presentation (e.g., backward, breech, and mal-positioned limbs or head) in uterus; hence, the head and legs cannot enter the dam’s birth canal. The insufficient maternal pelvic size also can induce the dystocia especially in beef heifers. For prevention of dystocia, dam’s genetic inheritance (e.g., adequate pelvic size and calving ease) should be taken into consideration for heifer selection (10), and frequent monitoring of calving cow is required for appropriate calving assistance (86).

**Immunity**

The bovine placenta does not allow passive transfer of antibody to fetus; hence, the newborn calf does not have any antibody from cow and is very susceptible to environmental pathogens. The resistance of the calf to the enteric disease closely relates to
the timely consumption of good quality and sufficient quantity of colostrum (8). The neonatal calf should ideally receive 1-2 liters of colostrum within the first 6 hours after birth (25). The composition of colostrum includes antibodies, immune cells (neutrophils, macrophages T cells and B cells), complements, lactoferrin, insulin-like growth factor-1, transforming growth factor, interferon and other soluble factors as well as nutrients (sugars and fat-soluble vitamins) (110). Immunoglobulin G is the primary antibody isotype in bovine colostrum (108).

The quality of colostrum varies based on calving number, nutritional status and vaccination of cow (117, 148). However, the calf born to a heifer can get an acceptable level of maternally derived immunity if it ingests enough volume of colostrum in the first 24 hours of life (86). Heifers have greater likelihood for dystocia, mis-mothering and poor colostrum in comparison to a multiparous cow (117). Therefore, other cow-calf management practices (e.g., calving heifers first and segregation of calves based on birth date) should also be considered for reducing the chance to get an infectious disease.

The primary function of colostrum is enhancement of the calf’s immune system through passive transfer of antibody against pathogens. Ideally, calves should receive colostrum from their dams; however with current production practices, colostrum is often mixed from several cows or purchased. One caution with colostrum feeding is transmission of BVDV, bovine leukemia virus and Johne’s disease which can be spread by infected or purchased colostrum (104, 159). In particular, *Mycobacterium avium paratuberculosis* (Johne’s disease) transmission is the number one risk factor when supplying the colostrum purchased from dairy cattle to beef cattle. Therefore, colostrum from dairy farm of unknown infection status should be avoided; it is recommended to get
supplemental colostrum produced from the farm of origin or from a historically disease-free farm.

If the farm has been suffering from specific pathogens such as BRV, BCoV, *C. perfringens* and *E. coli K99*+, vaccination of the dam could increase the colostrum quality with specific antibody against the targeted pathogens (25). Currently, commercial multivalent vaccines for these pathogens are available. Some of the vaccines can be given to the newborn calf orally before the calf uptakes colostrum while some of the vaccines are for cow vaccination. Most vaccines contain either modified-live or killed organisms or a combination of the two. For examples, ScourGuard® 4KC (Pfizer animal health) is a multivalent killed vaccine for *E. coli K99*+, BRV-A, BCoV and *C. perfringens* type C which is given to cows (134). Scour Bos™ 4 (NOVARTIS) is a bivalent killed vaccine against BRV and BCoV which is given to cows (112). CalfGuard® (Pfizer animal health) is a bivalent modified-live vaccine for BRV-A and BCoV which is given orally to newborn calves (133).

*Environmental stress and contamination*

Harsh weather conditions, such as cold temperatures, rain, heavy snow, wind and high moisture, act as stress factors to young calves and increase the susceptibility of calf to diarrhea (14, 86, 87). Neonatal calves are not able to regulate its body temperature well when exposed to extreme weather condition, which may induce hypothermia or hyperthermia resulting in impairment of immune system. The dam is less influenced by environmental stress than is calf. However, there is still increasing probability of dystocia or metabolic disease due to environmental stress (105). Special care is required to reduce
environmental risk factors closely related with calving season, including provision of dry, draft-free shelter (105). The calving season can be adjusted to more favorable environmental conditions by implementing a controlled breeding program.

The exposure to the contaminated environment is the main cause of calf diarrhea (93). A simple solution would be reducing of pathogen load into the environment where calves are raised although such a simple solution has always been a challenge for producers. After birth, calves are directly exposed to contaminated environments which can be influenced by various factors, such as the presence of infected animals, overcrowding, concurrent cow-heifer-calving, contaminated calving lots and no segregation of calves by age (86, 87). These factors usually work synergistically, which increase the opportunity for longer duration of exposure to higher quantity of pathogens. Conversely, intervention for calf diarrhea is conducted by focusing on the control and prevention of each factor (e.g., pathogens and environment contamination). The basic concepts of intervention for calf diarrhea are based on: 1) reduced pathogen exposure: planning to breed and calve to heifers first, which give less exposure of pathogens to the more susceptible newborn calves; 2) reducing the pathogen loading into environment: shortening calving season by scheduling breeding, which reduce the period of pathogen into environment; and 3) keeping a clean area (or pathogen-free area): animal can be grouped according to their calving date so that the calving area can be kept clean from the previous calving group.

The Sandhills Calving System has been reported to be highly effective for controlling calf diarrhea caused by multiple pathogens (153). The basic idea of the system focuses on preventing pathogen exposure at early stage by segregating groups of calves in
the order of calving and keeping a clean calving area. Briefly, a group of cows move into
the first calving pasture when calving begins and continues calving in the first pasture for 2
weeks. Cows that have not yet calved by the end of the second week are moved to the
second pasture where calving continues for 1 week. Any remaining cows not yet calved
are moved to the third pasture where calving continues for another 1 week. Finally, calve
born in different pastures get together when the youngest born calf becomes 4 weeks of
age. The calving interval in each pasture area can vary depending upon herd size,
available pasture and previous history of calf diarrhea of each farm.

Although the Sandhills Calving System management was initially introduced for
pasture calving cows, the concept is applicable to dry lot calving (e.g., concentrated cow-
calf operations found on many farms in Iowa) depending upon each farm’s situation. For
example, when the pasture area is not enough for rotational calving or segregation of cow-
calf, the corn field or soybean field can be utilized as calving lot or for isolation of sick
animal in lieu of the pasture area during the off-season (e.g., after harvesting or before
cultivation of crop).

**Conclusion**

Calf diarrhea has been a major disease problem in the US cattle industry. 
Economic impact of calf diarrhea to the industry is still significant, although many new
intervention strategies (e.g., vaccine, medications and herd management) have been
developed and practiced to minimize the economic loss. The persistence of the problem at
a significance level in the field may have been attributed to the multifactorial nature of calf
diarrhea, including permutations of infectious diseases, lack of clear understanding the
disease ecology, environmental hygiene and biased epidemiological data.

As described above, multiple infectious agents are involved in neonatal calf
diarrhea and are usually endemic infections on the farm. Genetic diversity, continuous
evolution and/or environmental ubiquity of pathogens are still impediments to effective
control of the disease. Use of highly sensitive diagnostic tests has increased the detection
frequency of pathogens which were previously neglected, suggesting that optimal and
appropriate diagnostic tests or platforms should be employed for detecting the target
pathogens accurately and timely with minimum bias of testing outcome. Non-infectious
risk factors should also be considered equally important as infectious factors due to the fact
that newborn animals are vulnerable to environmental stresses. Management and control
of disease should be focused on 3 points: a) characteristics of pathogens (e.g., pathogenic
mechanism, prevalence in field and genetic evolution); b) advantages and disadvantages of
various diagnostic methods and their application for diagnostic investigation; and c) cow-
calf management for disease prevention and control.

**STATEMENT OF PROBLEM AND SPECIFIC AIMS OF STUDY**

Calf diarrhea causes significant economic loss in the bovine industry due to the
treatment costs, labor costs, poor growth performance, high mortality and high morbidity.
A survey conducted in 2007 found that more than 50% of deaths in unweaned calves were
due to diarrhea. Calf scouring is a multi-factorial disease. Management, nutritional and
environmental factors, and/or infectious disease account for the majority of scouring problem. Related to infectious disease accurate diagnosis of causative agent(s) at early stage is critical for prevention and control. Multiple pathogens are known or postulated to cause or contribute to calf diarrhea. However, their significance and interaction in the disease has not been clearly addressed, not to mention uncertainty on the role of newly emerging viral pathogens such as bovine caliciviruses in calf diarrhea. It was also of interest how diagnostic testing influences such an assessment as biased epidemiological data can lead to a wrong perception on disease ecology in a population.

The long term goal of our project is to better understand the epidemiology, ecology and pathogenesis of well-known and potential bovine enteric pathogens in the field and develop better intervention strategies. Specific aims of the current study were: 1) develop highly specific and sensitive diagnostic methods for simultaneous detection of major bovine enteric pathogens; 2) determine the prevalence and molecular characteristics of bovine norovirus (BNoV) which has recently emerged as a potential enteric pathogen worldwide, in the US Midwest cattle farms; and 3) characterize the epidemiology of historically well-known and emerging bovine enteric pathogens in calf diarrhea.
REFERENCES


equine rotaviruses: identification of genotype G10,P6[1] and G1 strains and a new
VP7 genotype (G16) strain in specimens from diarrheic foals in India. J Clin

multiplex PCR assay for rapid detection and toxintyping of Clostridium perfringens

Lesions of gnotobiotic calves experimentally infected with a calicivirus-like

recombination between two genotypes of genogroup III bovine noroviruses
(BoNVs) and capsid sequence diversity among BoNVs and Nebraska-like bovine

60. Harding, M. J., X. Cao, H. Shams, A. F. Johnson, V. B. Vassilev, L. H. Gil, D.
Donis. 2002. Role of bovine viral diarrhea virus biotype in the establishment of

61. Haschek, B., D. Klein, V. Benetka, C. Herrera, I. Sommerfeld-Stur, S. Vilcek,
K. Moestl, and W. Baumgartner. 2006. Detection of bovine torovirus in neonatal


102. **McClane, B.** 2001. The complex interactions between Clostridium perfringens enterotoxin and epithelial tight junctions. Toxicon **39:**1781-91.


122. Park, J., S. Kurosawa, J. Watanabe, and K. Ishihara. 2004. Evaluation of 2-
methacryloyloxyethyl phosphorylcholine polymeric nanoparticle for immunoassay

Yang, S. K. Kim, M. I. Kang, and K. O. Cho. 2007. Molecular epidemiology of

characterization of unclassified bovine enteric caliciviruses in South Korea. Vet

and K. O. Cho. 2006. Detection and characterization of bovine coronaviruses in
fecal specimens of adult cattle with diarrhea during the warmer seasons. J Clin
Microbiol 44:3178-88.

enteric and respiratory tropisms of winter dysentery bovine coronavirus in calves.

and K. O. Cho. 2008. Molecular epidemiology of bovine toroviruses circulating in


134. **Pfizer.** ScourGuard® 4KC (Pfizer animal health) [https://animalhealth.pfizer.com/sites/pahweb/US/EN/Products/Pages/Scourguard_4KC.aspx](https://animalhealth.pfizer.com/sites/pahweb/US/EN/Products/Pages/Scourguard_4KC.aspx).


139. **Reis, B. P., S. P. Zhang, R. M. Tsolis, A. J. Baumler, L. G. Adams, and R. L. Santos.** 2003. The attenuated sopB mutant of Salmonella enterica serovar Typhimurium has the same tissue distribution and host chemokine response as the wild type in bovine Peyer's patches. Veterinary Microbiology **97:**269-277.


of the burden of illness caused by nontyphoidal Salmonella infections in the United


CHAPTER 2. DEVELOPMENT OF A PANEL OF MULTIPLEX REAL-TIME PCR ASSAYS FOR SIMULTANEOUS DETECTION OF MAJOR AGENTS CAUSING CALF DIARRHEA IN FECES


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ABSTRACT

Calf diarrhea is a major economic burden to the bovine industry. Since multiple infectious agents can be involved in calf diarrhea and the detection of each of the causative agents by traditional methods is laborious and expensive, a panel of two multiplex real-time PCR assays was developed for rapid and simultaneous detection of the five major bovine enteric pathogens (i.e., Bovine coronavirus [BCoV; formally known as Betacoronavirus 1], group A bovine rotavirus [BRV], Salmonella spp., Escherichia coli [E. coli] K99+, and Cryptosporidium parvum). The estimated detection limit (i.e., analytic sensitivity) of the panel was 0.1 TCID\textsubscript{50} for BCoV and group A BRV, 5 and 0.5 CFU for E. coli K99+ and Salmonella, respectively, and 50 oocysts for Cryptosporidium per reaction. In testing 243 fecal samples obtained from submissions to the Iowa State University Veterinary Diagnostic Laboratory or experimental animals with known infection status, the newly developed multiplex real-time PCR panel simultaneously detected all five pathogens directly from fecal samples and was more rapid and sensitive than the traditional
diagnostic tests. The PCR panel showed 89-97% agreement with those conventional
diagnostic tests, demonstrating the diagnostic sensitivity equal to or better than that of the
conventional tests. In conclusion, the multiplex real-time PCR panel can be a tool for a
timely and accurate diagnosis of calf diarrhea associated with BCoV, group A BRV, *E.
coli* K99+, *Salmonella*, and/or *Cryptosporidium*.

**INTRODUCTION**

Calf diarrhea causes major economic losses to the bovine industry due to high
mortality and morbidity. According to the 2007 National Animal Health Monitoring
System for U.S. dairy (http://nahms.aphis.usda.gov/dairy/index.htm), more than 50% of
deaths in unweaned calves were due to diarrhea. Although noninfectious factors, such as
insufficient uptake of colostrum, poor sanitation, stress, and cold weather, could cause
neonatal calf diarrhea, various infectious agents such as viruses, bacteria, and protozoa are
involved in calf diarrhea. The major infectious agents that have been implicated in calf
diarrhea are bovine coronavirus (BCoV), bovine rotavirus (BRV) group A, and bovine
viral diarrhea virus (BVDV) as viral agents; *Salmonella* spp., *E. coli* K99+, and
*Clostridium* spp as bacterial agents; and *Cryptosporidium* as a protozoan agent. Among
these, BCoV, group A BRV, *Salmonella* spp., *E. coli* K99+, and *Cryptosporidium parvum*
are known as the 5 most common pathogens identified in scouring calves less than 2
months of age.1,23,24,26
Bovine coronavirus is an enveloped virus with positive-sense, single-stranded RNA genome and is classified into genus *Betacoronavirus* as species *Betacoronavirus 1* along with mouse hepatitis virus, human coronavirus OC43, rat sialodacryoadenitis virus, porcine hemagglutinating encephalomyelitis virus, canine respiratory coronavirus, and equine coronavirus.\(^9,14\) Besides causing calf diarrhea, BCoV infection is also associated with winter dysentery in adult cattle as well as respiratory disease in all ages of cattle.\(^7,18\) 

BRV is a nonenveloped virus and possess 11 double-stranded RNA segments.\(^24\) Most BRV belong to group A rotavirus based on the antigenic similarity of the intermediate capsid protein (VP6), which is responsible for 95% BRV infection in the world, although groups B and C rotaviruses have also been identified in the field cases of BRV infection.\(^8,12,19,28\) Both BCoV and group A BRV are often detected concurrently in scouring calves.\(^4,24\) Infection with the viruses reduces the absorptive capacity of the intestines because of destruction of enterocytes and disrupts reabsorption of water, thereby leading to loss of fluid and electrolytes.\(^24\)

The diarrhea caused by *Salmonella* infection is characterized by watery and mucoid diarrhea with the presence of fibrin and blood.\(^11\) Even though *Salmonella* can cause diarrhea in both adult cattle and calves, infection is more common and often causes severe symptoms in 10-day to 3-month-old calves.\(^11\) On the other hand, *E. coli* K99\(^+\) causes a watery diarrhea, dehydration, and weakness in 1- to 4-day-old newborn calves.\(^3\) The fimbrial adhesion F5 (K99) promotes the attachment of bacterial cells to glycoproteins on the surface of epithelial cells of the jejunum and/or ileum, and bacterial enterotoxin also causes damage to the epithelial cells, resulting in fluid secretion and diarrhea.\(^2\)
In addition to the viral and bacterial agents described above, protozoan parasite, *Cryptosporidium*, also causes severe acute diarrhea in various animal species.\(^5\) *Cryptosporidium parvum* is the most important protozoa related to calf diarrhea in 1- to 4-week-old calves.\(^26\) The invasion of *Cryptosporidium* in enterocytes induces changes in the cell cytoskeleton, including the absence of microvilli and the shortening of columnar epithelial cells. These changes greatly contribute to the development of diarrhea due to malabsorption and fermentation of undigested milk in the intestinal lumen of young calves.\(^10\)

Since these various pathogens are individually or concurrently involved in calf diarrhea, differential diagnosis of these pathogens with rapid turnaround time is essential to implement appropriate treatment and preventive practice in a timely manner. Laboratory techniques that have been commonly used in veterinary diagnostic laboratories to identify these pathogens are virus isolation, electron microscopy, bacterial culture, fecal flotation method, antigen-capture enzyme-linked immunosorbent assay (Ag-ELISA), latex agglutination test (LAT), and/or polymerase chain reaction (PCR). These conventional methods are laborious, expensive, and/or slow in turnaround. Furthermore, they can be relatively insensitive depending on the quality and timing of sampling. As a rapid detection of these pathogens at the early stage of outbreak substantially contributes to minimizing the spread of infection and increasing treatment efficiency, a panel of two multiplex real-time PCR assays (hereafter, multiplex PCR panel), which can simultaneously detect the major causative pathogens (i.e., BCoV, group A BRV, *Salmonella* spp., *E. coli* K99\(^+\), and
Cryptosporidium) were developed, and their performance and utility in diagnosis of calf diarrhea was evaluated in this study.

MATERIALS AND METHODS

Reference strains of virus, bacteria, and parasites

Bovine coronavirus (Nebraska strain) was purchased from the USDA National Veterinary Services Laboratories (NVSL). Three different strains (NCDV, WC3, and B223) of group A BRV and two bacterial strains, E. coli K99\(^+\) (ATCC 31616) and Salmonella typhimurium (ATCC 14028), were purchased from American Type Culture Collection (ATCC). Cryptosporidium parvum (Iowa strain) was purchased from Waterborne Inc. These agents were used in assessing the analytic sensitivity of tests, in addition to serving as positive controls.

Specimens

Experimental specimens. Five calves were inoculated with 3 ml of Cryptosporidium parvum prepared at the rate of 500 oocysts/ml. A total of 30 fecal samples collected from the five calves at 0, 4, 8, 12, 16, and 20 days post challenge were kindly supplied by Dr. Jeffrey Knittel at Boehringer-Ingelheim Vetmedica Inc. (St. Joseph, MO, USA).

Clinical specimens. A total of 243 feces or intestinal contents, which were collected from diarrheic calves and submitted to the Iowa State University Veterinary Diagnostic
Laboratory (ISU-VDL) in 2007, were used to evaluate the performance of the multiplex real-time PCR panel in comparison to other laboratory procedures routinely used at ISU-VDL for the same target agents (i.e., real-time RT-PCR for BCoV, Ag-ELISA for rotavirus group A, bacterial culture and LAT for *E. coli* K99+, bacterial culture and serotyping for *Salmonella*, and microscopic observation with acid-fast staining for *Cryptosporidia*). In addition, 72 fecal samples collected from clinically healthy cattle in three dairy farms in Iowa were also used to validate the specificity of the multiplex real-time PCR panel.

**Nucleic acid extraction**

Nucleic acids of all target agents were simultaneously extracted from specimens by use of MagMax™ Total Nucleic Acid Isolation Kit as described in the manufacturer’s manual. Briefly, 0.01 M phosphate buffered saline (PBS, pH 7.4) was added to each sample to make 30% fecal homogenates. After centrifugation for 1 min at 100 x g to pellet larger-size particles, 175 µl of the supernatant of each sample was added to a bead tube containing zirconia beads and 235 µl of lysis/binding solution. The bead tube was beaten at 20 Hz for 5 min with TissueLyser. After the beating process, the bead tubes were centrifuged at 16,000 x g for 3 min, and the supernatant was carefully transferred into clean microcentrifuge tubes. After another centrifugation at 16,000 x g for 6 min, 115 µl of the supernatant was transferred to a 96-well, deep-well microplate which contained 20 µl of paramagnetic beads, and 65 µl of 100% isopropanol. The deep-well microplate and five additional 96-well plates (VWR) – 2 plates with 150-µl washing solution 1 per well, 2 plates with 150-µl washing solution 2 per well, and 1 plate with 50-µl elution buffer per well – were placed in KingFisher® 96 magnetic particle processor for automated
extraction process. The automated process consisted of lysis/binding step for 5 min; two-
time first washing step each for 90 sec; two-time second washing step each for 2 min and
30 sec respectively; dry step for 1 min; and, finally, the elution step for 3 min. The
extracted total nucleic acids in the elution plate were stored in -80°C until used for PCR
reaction.

Oligonucleotides

The sequence information of primers and probes used in the multiplex real-time
PCR panel are summarized in Table 1. Primers and probes for *E. coli K99*, *Salmonella*,
and *Cryptosporidium* were adopted from published information. The primers and probes
for BRV were designed in the current study. Three each of forward and reverse primers
and two probes were designed based on VP6 gene, which encodes intermediate capsid
protein, using Primer Express software\(^b\) to cover all group A BRV strains, whose VP6
sequences are available in GenBank (Fig. 1). All of the primers and probes were
synthesized and purchased from Integrated DNA Technologies (IDT)\(^i\) except Minor
Groove Binder probes for BCoV and BRV.\(^d\)

Multiplex real-time PCR panel

The multiplex PCR panel was optimized with AgPath-ID\(^{TM}\) Multiplex RT-PCR
Kit\(^d\) following manufacturer’s recommended protocol in a 25-µl reaction volume using 8
µl of extracted template. All primers and probes were prepared at 25-µM working
concentration, and equal volumes of primers and probes were mixed together for each
target agent. Two primers and one probe were mixed in a single tube for BCoV, *E. coli*
K99\(^+\), *Salmonella*, or *Cryptosporidium* (i.e., BCoV mix, K99 mix, *Salmonella* mix, and *Cryptosporidium* mix, respectively), while three each of forward and reverse primers and two probes were mixed together for group A BRV (i.e., BRV mix). Two PCR reactions were prepared: one for viral agents (BCoV and BRV) and the other for bacterial/protozoan agents (*E. coli K99\(^+\)*, *Salmonella*, and *Cryptosporidium*). The final concentration of each primer or probe was 0.2 \(\mu\)M. The PCR amplification was performed on the ABI 7500 Fast Real-Time PCR System.\(^d\) Cycling conditions were as follows: a) reverse transcription for 10 min at 45\(^\circ\)C (This step was omitted for bacterial/protozoan PCR); b) a 10-min activation step at 95\(^\circ\)C; and c) 35 cycles of 15 sec at 95\(^\circ\)C and 60 sec at 60\(^\circ\)C. Samples with a threshold cycle (Ct) of 35 cycles or less were considered positive.

**Internal control plasmid**

Full-length genomic DNA (1768 bp) of porcine circovirus-2 (PCV2), which has been detected only in swine, was amplified with PCV2-specific primers, which contain XhoI and BamHI enzyme sites: Xho-PCVF0 5’-

CTCGAGCTCGAGACCAGCGCACTTGGCGAAGC-3’; BamH-PCVR1768 5’-

GGATCCGGATCCAATACTTACAGCGCACTTCTTTGC-3’. The amplified PCR product was cloned into a pSK (+) vector\(^j\) using the incorporated restriction enzyme sites. Based on the information of PCV2 real-time PCR described previously,\(^22\) five nucleotides in the probe recognition site of the cloned PCV2 genome were substituted with the sequences that have not been identified in any of PCV2 isolates using QuikChange\(^®\) II site-directed mutagenesis kit.\(^j\) The sequence information of primers and probe for the internal control is described in Table 1. The internal control was added to either samples
(before extraction) or extracts (after extraction) at a predetermined concentration so that it could be detected between 32 and 34 cycles of amplification.

**Bovine Coronavirus real-time RT-PCR**

A real-time PCR which is routinely used to diagnose calf diarrheic cases at the ISU-VDL was employed. The BCoV real-time PCR was originally designed based on a previously reported BCoV gel-based PCR. The diagnostic performance of the real-time PCR had been validated and optimized by testing reference viruses and clinical samples in the comparisons with the gel-based PCR and BCoV antigen-capture ELISA at the ISU-VDL (Harmon, unpublished data, 2003). The PCR was performed according to the protocol which has been routinely used at the ISU-VDL. RNA was extracted from specimens with MagMAX™ Viral RNA Isolation Kit as described in the manufacturer’s manual. The PCR was performed with QuantiTect™ probe RT-PCR Kit in a 25-µl reaction volume using 5-µl extracted template. Primers and probe were the same as employed in the multiplex PCR (Table 1) and the final concentration of primers or probe was 0.4 or 0.2 µM, respectively. The PCR amplification was performed on Smart Cycler® II as follows: 50°C for 30 min, 95°C for 15 min, and 35 cycles of 94°C for 15 sec and 60°C for 60 sec. Samples with a threshold cycle (Ct) of 35 cycles or less were considered positive.
**Rotavirus group A antigen-capture ELISA**

Samples were assayed by Premier™ Rotaclone® Kit following the procedures recommended by the manufacturer. Samples with optical density $\geq 0.3$ at 450 nm were considered positive for rotavirus.

**Isolation and identification of Salmonella sp.**

Samples were inoculated in Tetrathionate Broth and incubated for 24 hr at 42°C for enrichment. The enriched samples were plated on Brilliant Green Agar with Novobiocin, Hektoen Enteric Agar, and/or XLT4 Agar and then incubated at 35-37°C for 24 hr. Suspect *Salmonella* were subcultured from Brilliant green (pink colonies), Hektoen-enteric (green or black colonies), or XLT4 (black colonies) and confirmed as *Salmonella* in Kliglers, Urea, Sims, and Lysine agars and in the Trek Sensititre® Gram-Negative Identification panel. Colonies were serogrouped with commercial antisera in ISU-VDL or serotyped at the NVSL (Ames IA, USA) for final identification.

**Isolation and identification of E. coli K99**

Samples were plated directly onto Tergitol 7 Agar with triphenyltetrazolium chloride and incubated aerobically at 35-37°C for 24 hr. *E. coli* colonies with rough, intermediate, smooth, or mucoid morphology were subcultured to conventional biochemical tube media for identification. Kligler’s Iron Agar, Sims Agar, and Urea agar slants were inoculated and incubated aerobically at 35-37°C for 24 hr. The reactions were read and then compared to charts to confirm the identification. *E. coli* growth from direct
plating or from pure culture plating to E-agar™ was tested for the presence of K99 pilus antigen using the Piltest™ kit.¹

**Identification of Cryptosporidium by acid-fast staining**

Each sample was smeared on a glass slide, and the smear was dried briefly in ambient temperature. The air-dried smear was fixed in methanol for 10 min and then placed in Carbol Fuchsin' for 1–2 hr. The smear was washed with tap water for 1 min and placed in 1% acid alcohol until no more red color ran off. After washed briefly with tap water, the smear was counterstained in 0.5% Fast Green for 1 min. The slide was read under a light microscope after brief steps of washing and drying. Red and halo-shaped oocysts were identified as cryptosporidia.¹⁵

**Analysis of discrepant test results**

Samples with discrepant results between the multiplex real-time PCR and other traditional tests were re-tested by the multiplex PCR after re-extraction and further analyzed using sequencing and gel-based PCR for each target agent. In addition, reference BCoV, *E. coli* K99⁺ strains or PCV2 internal control plasmid were spiked in discrepant samples and tested again by the multiplex PCR to determine that negative PCR results were not due to PCR inhibition during the extraction or amplification procedures.

*Sequencing of real-time PCR products.* Real-time PCR products were electrophoresed and visualized on 2% gels to confirm the presence of specific amplified target gene with predicted molecular size. The amplicons were then purified using QIAquick® PCR purification kit² and submitted to the ISU Nucleic Acid Facility for
sequencing. The same amplification primers described in table 1 were used for sequencing of each target agent. Nucleic acid sequences of the PCR products were aligned with known sequences of the corresponding agents and analyzed using Lasergene® MegAlign software.

*Gel-based PCR tests.* Gel-based PCR for the 5 pathogens were optimized using QIAGEN® One-Step RT-PCR kit. The primers used to detect each agent are listed in Table 2. Cycling conditions of PCR were as follows: a) RT for 30 min at 50°C; b) a 15-min activation step at 95°C; c) 40 cycles of 30 sec at 94°C, 60 sec at 55°C, and 60 sec at 72°C; and d) final extension for 7 min at 72°C. RT enzyme mix and RT step were omitted for the PCR reactions of *E. coli K99*+, *Salmonella*, and *Cryptosporidium*.

**Statistical analysis**

The performance of the multiplex PCR was compared by calculating the percent agreement with other test results and the κ value as follows:

\[
\text{% Agreement} = \left[ \frac{\text{Agreed Pos + Neg}}{\text{Total tests (n=243)}} \right] \times 100;
\]

\[
\kappa = \frac{\Pr(a) - \Pr(e)}{1 - \Pr(e)}
\]

where \( \Pr(a) \) is the relative observed agreement between tests, and \( \Pr(e) \) is the probability that agreement is due to chance. If the tests are in complete agreement, \( \kappa = 1 \). If there is no agreement between the tests, then \( \kappa \leq 0 \). The interpretation of \( \kappa \) value was based on the guide provided by Landis and Koch: 16 Poor agreement (\( \kappa = 0.00 \)); slight (\( 0.01 < \kappa < 0.20 \)); Fair (\( 0.21 < \kappa < 0.40 \)); Moderate (\( 0.41 < \kappa < 0.60 \)); Substantial (\( 0.61 < \kappa < 0.80 \)); Almost perfect (\( 0.81 < \kappa < 1.00 \)).
RESULTS

Optimization of multiplex real-time PCR panel

All reference strains of the five agents with known virus titer (TCID$_{50}$/ml), number of bacterial colony (CFU/ml), or number of oocysts (per ml) were serially diluted 10-fold and used to optimize the multiplex PCR. The multiplex PCR panel simultaneously detected all of those reference strains, yet only specific target agents without any false-positive result. Standard curves were generated using the 10-fold serial dilutions with correlation coefficients ranging from 0.987 to 0.996 and slopes of 3.06 to 3.85 (Fig. 2). The limits of detection (i.e., analytic sensitivity) for each agent are 0.1 TCID$_{50}$ for BCoV and BRV, 5 and 0.5 CFU for *E. coli* K99$^+$ and *Salmonella*, respectively, and 50 oocysts for *Cryptosporidium* per reaction.

As a next step, the performance of the multiplex PCR and simplex PCR for each of 5 agents was directly compared on the same 96 extracts to determine any negative effect of multiplexing on the PCR detection. The test results by both simplex and multiplex PCR were completely matched and Ct differences between the both PCR reactions were not statistically significant (P > 0.1), demonstrating that multiplexing did not cause significant negative effect on the sensitivity or specificity of the PCR reactions. In addition, 72 fecal samples collected from clinically normal cattle were tested by routine bacterial culture and the multiplex PCR panel. Only coliform bacteria were isolated from the culture and all the samples were negative by the PCR panel for all 5 agents, suggesting that the PCR does not detect normal flora as any of the target agents.
When the multiplex PCR panel was run on 30 fecal samples collected from calves experimentally challenged with Cryptosporidium parvum, no amplification signal for Cryptosporidium was detected at 0 day post challenge (dpc), and yet the highest number of oocyst (10^{5.3}–10^{7.2} oocysts/ml) was detected in the fecal samples collected from the five calves at 4 or 8 dpc. At 20 dpc, 10^{3.7} oocysts were still detected from one calf (Table 3). No other agent (i.e., BCoV, group A BRV, E. coli K99+, Salmonella spp.) was detected from these fecal samples by the PCR panel.

**Performance of multiplex PCR panel in comparison with other tests**

Comparisons of test results on 243 scour samples between the multiplex PCR panel and other laboratory tests routinely used at ISU-VDL for the 5 target agents are summarized in Table 4. Among all of the examined samples, the multiplex PCR panel detected the BCoV genome in 54 samples, which was 12 more than the number of positive samples identified by BCoV real-time RT-PCR (n = 42), whereas all the other samples (n = 189) were negative for BCoV by both the PCR tests. Accordingly, the test agreement between BCoV RT-PCR and multiplex PCR was 95% (231/243), and κ value was 0.844. In the case of BRV detection, the multiplex PCR identified 23 more samples as positive for BRV compared with the rotavirus Ag-ELISA, whereas 2 positive samples by the ELISA were negative for BRV by the multiplex PCR. The test agreement between two tests was 89% (218/243), and κ value was 0.733.

In comparison with bacterial culture methods for two bacterial pathogens (i.e., Salmonella and E. coli K99+), the multiplex PCR identified four more positive samples for Salmonella sp. compared with the culture results, whereas 3 positive samples by culture
were negative by the multiplex PCR. The agreement between the multiplex PCR and *Salmonella* culture was 97% (236/243), and κ value was 0.887. In the case of *E. coli* K99+, the multiplex PCR detected 39 positive samples, which were 9 more than the number of positive samples identified by culture and LAT. However, 5 positive samples by the culture method followed by LAT were negative by the multiplex PCR. The agreement of the two tests was 94% (229/243), and κ value was 0.776.

The multiplex PCR was also compared with microscopic observation with acid-fast staining for the detection of *Cryptosporidium* spp. in the samples. The PCR detected 14 more positive samples than those by the microscopic observation, while two samples identified as positive for *Cryptosporidium* spp. by the microscopic observation were negative by the PCR for *Cryptosporidium parvum*. The test agreement between the multiplex PCR and microscopic observation was 93% (227/243), and κ value was 0.756.

**Analyses of discrepant samples**

All of the samples (n = 12) that were negative by the multiplex PCR panel, but positive for any of the five target agents by other conventional tests (Table 3), were retested by the multiplex PCR and gel-based PCR after re-extraction. The same results as initially observed were reproduced on both PCR assays. When reference BCoV, *E. coli* K99+ strains, or internal control plasmid at a known amount were spiked in those samples and tested, the spiked virus, bacteria, or internal control were detected as expected, confirming that the negative PCR results were not attributed to the presence of inhibitory substances in the samples. Nonetheless, the *E. coli* K99+ (n = 5) and *Salmonella* isolates (n
= 3) were cultured from the samples and all isolates were confirmed to be *E. coli K99* or *Salmonella* by the multiplex PCR.

For the samples (n = 62) that were positive for any of the target agents by the multiplex PCR, but negative by other conventional tests [BCoV (n = 12), BRV (n = 23), *Salmonella* (n = 4), *E. coli K99* (n = 9), or *Cryptosporidium* (n = 14)], the simplex PCR and gel-based PCR for each agent were repeated, revealing the same results as those by the multiplex PCR. Each of the PCR products had the expected molecular size for each agent (Fig. 3). The sequences from the PCR products shared 98-100% homologies with the target amplification regions for each agent.

**DISCUSSION**

A multiplex real-time PCR panel that can simultaneously detect five major causative agents of calf diarrhea (i.e., BCoV, BRV, *Salmonella*, *E. coli K99*, and *Cryptosporidium parvum*) was developed in this study. As the new multiplex PCR can test 96 samples to determine the presence and absence of five different viral, bacterial, and protozoan agents within 4 hr, it can greatly reduce the cost, labor, and turnaround time compared with various routine diagnostic techniques, such as individual PCR, bacterial culture, serotyping, LAT, microscopic examination, and Ag-ELISA. Since fecal material is one of most difficult matrices from which to conduct PCR and extraction is the most labor-intensive and expensive procedure in PCR performance, several innovative approaches were made in the multiplex PCR to save amount of time, labor and cost. First, the PCR
employed a unique extraction method (i.e., Total Nucleic Acid Isolation Kit) which can simultaneously purify either RNA or DNA of viral, bacterial, and protozoan agents directly from fecal materials in a single tube. Second, a magnetic particle processor in a 96-well format (e.g., KingFisher® 96) was used in the extraction process to minimize inhibition problem from feces and make the test fit for high throughput, thus substantially reducing the extraction time (<1.5 hr for 96 samples). Third, the PCR reaction was done using one-step PCR procedure and in a fast gene amplification system (i.e., ABI 7500 Fast) which permits PCR to be completed within 2.5 hr. Any of these is applicable individually or in combination to development of other real-time PCR based multiplex or panel testing which becomes a common practice since disease problems in modern animal agriculture tend to be multifactorial.

Overall, a good agreement in the test results was observed between the multiplex PCR panel and the traditional diagnostic methods, ranging between 89% and 97% based on the test results of 243 clinical samples. Most of the discrepant results between the multiplex PCR and the traditional tests (62 out of 74 total discrepant samples) were due to the higher sensitivity of the multiplex PCR panel, since the positive results of 62 samples for either of the 5 agents by the PCR panel could be confirmed with respective gel-based PCR tests or sequencing of the PCR products. The other 12 discordant samples, which were positive by the traditional tests, but negative by the multiplex PCR panel, were still negative by the respective gel-based PCR tests, suggesting that there was no detectable target gene in the samples. Reference BCoV, *E. coli* K99+ strain, or internal control plasmid spiked in those samples were successfully detected by the PCR panel, ensuring
that the negative PCR results were not due to gene degradation or PCR inhibition during the extraction or amplification procedures. The possible explanations for the negative result of the multiplex PCR on these samples include the following: 1) false-positive results of the traditional diagnostic assays due to nonspecific detection or contamination, 2) larger volume of the samples (up to 100 times more) used for the tradition assays compared with that used for the multiplex PCR, and 3) atypical viruses or bacteria that have sequence substitutions at the primer or probe recognition sites.

BCoV real-time PCR employed in the multiplex PCR panel has been developed based on a nested PCR previously described by Cho et al. and being routinely used to detect BCoV from calf diarrheic cases at ISU-VDL. The test results of the real-time PCR have shown good agreement with those of the nested PCR and correlated well with pathological evidence and clinical history. The primers and probe for group A BRV were designed in this study because no real-time RT-PCR that can detect a broad range of genetically diverse group A BRV has been reported previously. Inclusion of three pairs of forward and reversed primers and two probes, which were designed using the VP6 gene of BRV, was necessary to cover all known group A BRV based on the sequence database in GenBank since the sequence homology of even VP6, which is highly conserved gene of BRV, ranged from 86 to 96% among group A BRV. The multiplex PCR appeared to detect most, if not all, of field BRV strains because it detected BRV virus in the 50 field samples that were also positive by a group A rotavirus Ag-ELISA kit, which is known to detect all group A rotavirus of various animal species including humans, although both the multiplex and gel-based rotavirus PCR tests could not detect BRV in 2 samples that were positive by
the ELISA. Presuming that the result of Ag-ELISA on those 2 samples were correct, it emphasizes the need for periodical updating of primers and/or probes to ensure that the PCR detects all circulating field strains as RNA viruses are known to evolve rapidly. Another important point to carefully consider when interpreting the PCR results for BCoV and BRV is vaccination history as oral vaccination with attenuated live BCoV and BRV vaccines are sometimes applied to newborn calves even though cow vaccination seems to be more commonly practiced in many bovine herds. Using electron microscopy, both BCoV and BRV were detected in feces until 3 and 7 days, respectively, after experimental vaccination with a BCoV-BRV multivalent attenuated vaccine via oral route. Therefore, there is a great chance that the multiplex PCR detects both wild-type and vaccine viruses if attenuated vaccines are applied to newborn calves and samples are collected within such a timeframe.

The *Salmonella* PCR employed in the current multiplex PCR panel was reported to detect *Citrobacter amalonaticus* as *Salmonella* false positively, whereas other *Citrobacter spp.* were not detected by this PCR. Nonetheless, *Citrobacter amalonaticus* did not seem to cause a significant problem in bovine, because the 72 fecal samples collected from healthy cattle were negative for *Salmonella* by the multiplex PCR, and only *Citrobacter freundii* were isolated from two samples out of 243 clinical samples by bacterial culture; however, those isolates were negative for *Salmonella* by PCR, suggesting that *Citrobacter amalonaticus* is not commonly detected in bovine species.

In conclusion, the new multiplex PCR panel is specific and more sensitive than other traditional diagnostic methods, and drastically decreases turnaround time, labor, and
cost. Therefore, this PCR panel will make diagnosticians be able to rapidly determine the causative agents for bovine diarrhea in the early stages of disease and help practitioners initiate appropriate treatments or interventions quickly.

ACKNOWLEDGEMENTS

The authors thank to Dr. Subhashinie Kariyawasam at Pennsylvania State University (State College, PA) for providing K99⁺ *E. coli* PCR protocol. Dr. Jeffrey Knittel at Boehringer-Ingelheim Vetmedica Inc. (St. Joseph, MO) is acknowledged for providing *Cryptosporidium*-challenged samples which were very valuable to this study. The authors are also grateful to Drs. Vickie Cooper, Bruce Leuschen, Terry Engelken, and Leo Timms at Iowa State University for advice in test evaluation. The study was supported in part by funding from Iowa Calf Scour Fund.

SOURCE AND MANUFACTURES

a. USDA National Veterinary Services Laboratories, Ames, IA.

b. American Type Culture Collection, Manassas, VA.

c. Waterborne Inc., New Orleans, LA.

d. Ambion/Applied Biosystems, Austin, TX.

e. Qiagen, Valencia, CA.
f. VWR, West Chester, PA.

g. Thermo Scientific, Waltham, MA.

h. Version 3.0, Applied Biosystems, Foster City, CA.

i. Integrated DNA Technologies, Coralville, IA.

j. Stratagene, La Jolla, CA.

k. Cepheid, Sunnyvale, CA.

l. Meridian Bioscience, Cincinnati, OH.

m. Difco, Sparks, MD.

n. Remel, Lenexa, KS.

o. Trek Diagnostic Systems, Cleveland, OH.

p. Becton Dickinson; or Statens Serum Institute, Copenhagen, Denmark.

q. VMRD, Pullman, WA.

r. Newcomer supply, Middleton, WI.

s. DNASTAR Inc., Madison, WI.

REFERENCES


<table>
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<th>Agent (target gene)</th>
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<td>GCCATGCTGTTGATGAT</td>
<td>129</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Stn-rev</td>
<td>GTTACCAGTAGCCGGAAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stn-probe (Cy5/BHQ)</td>
<td>TTTTGCACCACCGCAAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptosporidium (COWP)</td>
<td>Crypto-fwd</td>
<td>CAAATTGATACCGTTTGTCCTTCT</td>
<td>151</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Crypto-rev</td>
<td>GCCATGTCATCCATTGACGGGCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Crypto-probe (JOE/BHQ)</td>
<td>TGCATACATTTGTCCTGACAAAATTGAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Internal control</td>
<td>P1570</td>
<td>TGCCCGGCAGTATCTGATT</td>
<td>73</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>P1642</td>
<td>CAGCTGCCAGCGGCTTGGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P1591M (Cy3/BHQ)</td>
<td>CCTCGAATCAAACGCGGGCTTTGGAATG</td>
<td></td>
<td>Current study</td>
</tr>
</tbody>
</table>
Table 2. The nucleotide information of primer used for alternative gel-based PCR.

<table>
<thead>
<tr>
<th>Agent (target gene)</th>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Product size (base pair)</th>
<th>Reference no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine coronavirus (N)</td>
<td>BCoVF</td>
<td>CCGATCAGTCCGACCAATC</td>
<td>406</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>BCoVR</td>
<td>AGAATGTCAAGCGGGGAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine rotavirus group A (VP6)</td>
<td>BRVF</td>
<td>ACCACCAATATGACACCAGC</td>
<td>294</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>BRVR</td>
<td>CATGCTTCTAATGGAAGCCAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli K99</em>+ (K99)</td>
<td>K99F</td>
<td>GCGACTACCAATGCTTCTGCGAATAC</td>
<td>230</td>
<td>Current study</td>
</tr>
<tr>
<td></td>
<td>K99R</td>
<td>GAACCAGACCAGTCAATACGAGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salmonella (16S rDNA)</td>
<td>16SF</td>
<td>TGGTTGTTAATAACCGCA</td>
<td>574</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>16SR</td>
<td>CACAAATCCATCTCTGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptosporidium (CWP)</td>
<td>BB-3F</td>
<td>GCGAAGATGACCTTTGTATTTG</td>
<td>194</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>BB-4R</td>
<td>AGGATTTCTTCTTGAGGTTC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Detection of Cryptosporidium parvum by multiplex PCR performed on the fecal samples collected from calves experimentally challenged with Cryptosporidium parvum at 500 oocysts/ml.

<table>
<thead>
<tr>
<th>ID</th>
<th>Days post challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>526</td>
<td>Neg</td>
</tr>
<tr>
<td>530</td>
<td>Neg</td>
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<tr>
<td>537</td>
<td>Neg</td>
</tr>
<tr>
<td>538</td>
<td>Neg</td>
</tr>
<tr>
<td>540</td>
<td>Neg</td>
</tr>
</tbody>
</table>

* Log$_{10}$ of the number of oocysts/ml in the samples
Table 4. Comparative performance of multiplex PCR and traditional diagnostic assays in detecting Bovine coronavirus (BCoV), group A Bovine rotavirus (BRV), *Salmonella* spp., *Escherichia coli* K99*, or Cryptosporidium parvum* from fecal samples.

<table>
<thead>
<tr>
<th>Traditional tests</th>
<th>Multiplex PCR</th>
<th>% agreement (κ value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>BCoV real-time RT-PCR</td>
<td>42</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>189</td>
</tr>
<tr>
<td></td>
<td>54</td>
<td>189</td>
</tr>
<tr>
<td>Rotavirus group A antigen-capturing ELISA</td>
<td>50</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>73</td>
<td>170</td>
</tr>
<tr>
<td><em>Salmonella</em> culture and serotyping</td>
<td>33</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>203</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>206</td>
</tr>
<tr>
<td><em>E. coli</em> culture and latex agglutination for K99</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>199</td>
</tr>
<tr>
<td></td>
<td>39</td>
<td>204</td>
</tr>
<tr>
<td>Microscopic observation with acid fast staining for <em>Cryptosporidium</em></td>
<td>31</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>196</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>198</td>
</tr>
</tbody>
</table>

* BCoV = Bovine coronavirus; PCR = polymerase chain reaction; RT-PCR = reverse transcription polymerase chain reaction; Ag-ELISA = antigen-capture enzyme-linked immunosorbent assay; LAT = latex agglutination test.
Figure 1. Genetic variation of VP6 genes among Group A Bovine rotavirus. Each virus is indicated with strain name and GenBank accession number. The phylogenetic tree was constructed by neighbor-joining method. The reliability of analysis was determined by 1000 times repeated bootstraps.
Figure 2. Multiplex detection of group A Bovine rotavirus (BRV), Bovine coronavirus (BCoV), *Salmonella* sp., *E. coli* K99+, and *Cryptosporidium parvum*. All of the five agents with known virus titer (TCID$_{50}$/ml), number of bacterial colony (CFU/ml), or number of oocysts (per ml) were mixed and serially diluted 10-fold for simultaneous detection by multiplex PCR. Y axis indicates cycle threshold (Ct) values. Each regression line was constructed based on three repeated measurements.
**Figure 3.** Electrophoretic analysis of PCR products from group A bovine rotavirus (Ro), bovine coronavirus (Co), *Salmonella* sp. (S), *E. coli* K99\(^+\) (E) and *Cryptosporidium parvum* (Cr) on 2% agarose gel. Extraction was made with MagMAX Total Nucleic Acid Isolation Kit (Ambion/Applied Biosystems). PCR amplification was attempted with primers designed for multiplex real-time PCR and gel-based PCR as summarized in Tables 1 and 2, respectively.
CHAPTER 3. EVALUATION OF A COMMERCIAL RAPID TEST KIT FOR DETECTING BOVINE ENTERIC PATHOGENS IN FECES

A paper was published in Journal of Veterinary Diagnostic Investigation 24:559-562, 2012

Yong-II Cho, Dong Sun, Vickie Cooper, Grant Dewell, Kent Schwartz, Kyoung-Jin Yoon

ABSTRACT

Recently a commercial antigen-capturing ELISA kit in form of a dipstick (Bovine Enterichek, Biovet®) was made available to bovine practitioners and producers for the rapid detection of bovine coronavirus (BCoV), bovine rotavirus A (BRV-A), *Escherichia coli* (E. coli) K99+, and *Cryptosporidium parvum* (C. parvum) in feces from diarrheic calves. The diagnostic performance of Enterichek was evaluated in comparison with a multiplex real-time polymerase chain reaction assay (mrtPCR). One hundred fecal samples were procured from diagnostic submissions to Iowa State University Veterinary Diagnostic Laboratory and were used for the assessment. The agreement (i.e., κ value) in results for each pathogen between Enterichek and mrtPCR were 0.095 (BCoV), 0.521 (BRV-A), 0.823 (E. coli K99+), and 0.840 (C. parvum). In comparison to mrtPCR, the diagnostic sensitivity of Enterichek was 60.0%, 42.3%, 71.4% and 81.5%; and the diagnostic specificity was 51.4%, 100%, 100%, and 98.6% for BCoV, BRV-A, E. coli K99+, and C. parvum, respectively. This study suggested that Bovine Enterichek kit can be a rapid test tool in the field for detection of C. parvum or E. coli K99+ in feces from
calves at acute stage of clinical disease. However, BCoV positive and BRV-A negative results by the kit may need to be interpreted with caution due to their relatively low specificity and sensitivity.

**INTRODUCTION**

Calf diarrhea is a worldwide issue in cattle industry and causes a high rate of mortality and morbidity. Numerous pathogens [e.g., bovine coronavirus (BCoV), bovine rotavirus (BRV), bovine viral diarrhea virus, bovine norovirus, bovine torovirus, *Escherichia coli* (*E. coli*) K99*, *Salmonella* spp, *Cryptosporidium parvum* (*C. parvum*) and coccidia] have been reported to be associated with diarrhea in calves. Timely prevention and control of calf diarrhea is important to reduce economic losses to producers and improve animal welfare. Dealing with such a large number of potential etiological agents as well as various management factors (e.g., housing, colostrum uptake, herd size, and environmental temperature) is an ongoing challenge for effective control of enteric disease in newborn calves. Accurate and rapid confirmation of etiology early in the disease outbreak can aid in quick implementation of appropriate interventions to decrease losses. An animal-side or pen-side rapid test kit which is designed to simultaneously detect multiple pathogens is useful in the field.

Recently, a commercial ‘dipstick’ antigen-capturing ELISA (“Bovine Enterichek” kit; hereafter, Enterichek) has been marketed for rapid detection of 4 major bovine enteric pathogens [BCoV, BRV type A (BRV-A), *E. coli* K99*, and *C. parvum*] in feces from
diarrheic calves. The principle of the kit is based on a lateral flow immunochromatography assay\textsuperscript{1} which captures target antigen(s) within a fecal sample. The kit is designed to be a rapid test and can be used as animal-side or pen-side test in the field. Diagnostic sensitivity of the kit stated by the manufacturer is 63.6\%, 96.0\%, 82.6\%, and 78.3\% for BCoV, BRV-A, \textit{E. coli} K99\textsuperscript{+} and \textit{C. parvum}, respectively, while the stated diagnostic specificity of the kit is 97.4\%, 100.0\%, 94.4\% and 93.3\% for the same agents. However, no independent evaluation of the kit has been reported. Manufacturer’s internal evaluation of kit performance was comparing various tests for each of the different agents. Different laboratory methods have varying sensitivity and specificity for the specific targets for which they are designed. Use of different laboratory methods to validate test performance for the different agents can yield biased results due to varying degree of sensitivity and/or specificity among assays and unintended diagnostic error during testing.

The following study was conducted to assess the diagnostic performance of Enterichek in comparison with a multiplex real-time polymerase chain reaction (mrtPCR) which was designed to detect the same pathogens\textsuperscript{3} and is currently offered by the Iowa State University Veterinary Diagnostic Laboratory (ISUVDL) for detecting major bovine enteric pathogens in bovine feces.
MATERIALS AND METHODS

Study design

One hundred fecal samples were procured from submissions to ISUVDL during 2010. All samples were collected from diarrheic calves. No more than 3 fecal samples were collected from the same herd. All of samples were tested by both mrtPCR and Enterichek kit concurrently. The diagnostic performance of the Enterichek kit was evaluated by comparison of results to those of mrtPCR. The discrepant test results between the two tests were resolved by performing gel-based PCR or RT-PCR for each agent and sequencing PCR products as previously described.³

Bovine Enterichek assay

The kit includes strips and dilution tubes (Fig. 1). Each dilution tube has a lid with measuring spoon attached and contains 2 ml of a proprietary diluent. The kit was used to test samples as directed by the manufacturer with a modification. In brief, a spoonful amount (approximately 0.25 g) of sample was taken from each feces, transferred to a dilution tube, and mixed well with the diluent by shaking the tube. The diluted and homogenized feces in the dilution tube were then transferred to a container with larger opening instead of keeping them in the dilution tube so that all 4 strips could be dipped in the sample together at the same time without touching each other (Fig. 2A). The strips were hung together by a paper clip and were kept immersed in the liquid phase of the sample for approximately 10 min or until the liquid reached the top of each strip as recommended by the manufacturer. The strips were then removed from the sample and
kept for 5 min at room temperature for drying before reading. The sample was considered positive or negative for each target agent if the corresponding strip had two or one line, respectively. Testing was considered invalid if no line was observed on the strip.

**Bovine enteric PCR panel for detection of BCoV, BRV, *E. coli K99*⁺ and *C. parvum***

The mrtPCR was performed as previously described.³ All fecal samples were prepared with 0.01M phosphate-buffered saline (pH 7.4) for 30% fecal homogenates. After centrifuging the fecal homogenates for 1 min at 100 × g, 175 µl of the supernatant was used to extract genomic material of target agents using a commercial nucleic acid isolation kit according to the manufacturer’s instruction. A multiplex qPCR kit was used for the one-step RT-PCR. For PCR, 8 µl of nucleic acid template was mixed with 17 µl of reaction mixture containing primers and probes (200nM each), multiplex RT-PCR buffer, multiplex enzyme mix, and nuclease-free water. Amplification of the template was performed using an automated real-time PCR system. The cycling condition were as follows: reverse transcriptase reaction for 10 min at 45°C (skipped for DNA pathogens) and 10 min activation for the DNA polymerase, followed by 40 cycles of denaturation at 94°C for 10 sec and annealing/extension at 60°C for 60 sec. Samples with threshold cycle (Ct) ≤35 were considered positive for the corresponding target agent(s).

**Detection limit**

For each pathogen (i.e., BCoV, BRV-A, *E. coli K99*⁺, and *C. parvum*), 4 positive fecal samples as determined by mrtPCR were prepared from clinical cases. Each was
assessed for level of the target pathogen by mrtPCR using standard curves generated based on known copy numbers of plasmid standards constructed to contain the target gene of each pathogen. The fecal samples were then diluted by serial 2-fold dilution technique in the diluent provided with the Bovine Enterichek kit. Each of the diluted fecal samples was tested by both Enterichek and mrtPCR to determine the detection limit of Enterichek for each pathogen. The level (genomic copy # per ml) of the target pathogen in each diluted feces was determined based on Ct value using a standard curve generated from a set of varying copy numbers of plasmid constructed to contain the target gene of the pathogen.

Data analyses

The performance of Enterichek was compared with that of mrtPCR by using Chi-square analysis and κ calculation. The diagnostic sensitivity and specificity of the kit was then calculated. The κ value was interpreted as one of the following: poor (κ=0), slight (0.01<κ<0.20), fair (0.21<κ<0.40), moderate (0.41<κ<0.60), substantial (0.61<κ<0.80) and excellent (0.81 <κ<1.00).

RESULTS

Of the 100 samples tested by mrtPCR, 34% were positive for one of the 4 target pathogens, 45% were positive for more than one target pathogen, and the remaining (21%) were negative for all of the 4 target pathogens.

The results and comparative performance of Enterichek on the same 100 fecal samples in comparison to mrtPCR are summarized in Table 1. The agreement between
Enterichek and mrtPCR was excellent (98% and 94%, respectively) in detecting \textit{E. coli K99}\textsuperscript{+} and \textit{C. parvum}, acceptable (85%) in detecting BRV, and relatively poor (54%) in detecting BCoV. Accordingly, \(\kappa\) values were in a similar pattern: poor for BCoV, moderate for BRV-A, and excellent for \textit{E. coli K99}\textsuperscript{+} and \textit{C. parvum}. Samples with discrepant results between Enterichek and mrtPCR were re-tested by conventional gel-based PCR for each agent and sequencing PCR product. In all discrepant samples, the results of gel-based PCR and sequencing confirmed the results of mrtPCR.

The estimated detection limits of Enterichek for all agents were approximately 300 copies per 1ml. When mrtPCR was used as the standard, the diagnostic sensitivity of Enterichek was 60.0%, 42.3%, 71.4% and 81.5% for detection of BCoV, BRV-A, \textit{E. coli K99}\textsuperscript{+} and \textit{C. parvum}, respectively. The diagnostic specificity of Enterichek was 51.4%, 100%, 100% and 98.6% for detection of BCoV, BRV-A, \textit{E. coli K99}\textsuperscript{+} and \textit{C. parvum}, respectively.

**DISCUSSION**

Multiple factors, both infectious and non-infectious, are involved in calf diarrhea outbreaks, which makes disease control on farms difficult.\textsuperscript{10,11} Rapid and accurate diagnosis of various pathogens is essential for timely implementation of appropriate intervention or preventive measures in the herd to reduce economic losses.\textsuperscript{8} In this regard, an ‘animal-side’ or ‘pen-side’ rapid test kit is highly desirable as long as the kit has an appropriate and predictable level of sensitivity and specificity.
The current study was to evaluate diagnostic performance of a commercially available lateral flow chromatography-based rapid antigen detection kit (“Bovine Enterichek”). When compared to mrtPCR, the performance of Enterichek on fecal samples from clinical cases was comparable in detection of *E. coli* K99+ and *C. parvum* (κ value >0.8), suggesting that the kit can be a rapid test for these 2 agents in the field. The kit was, however, less than optimal in detecting BRV-A and BCoV in feces from clinical cases.

The low agreement (κ = 0.521) between mrtPCR and Enterichek for BRV-A was due to lower sensitivity (42.3%) of Enterichek than that of mrtPCR. The poor agreement (κ = 0.095) between mrtPCR and Enterichek for BCoV was due to both low sensitivity (60%) and low specificity (51.4%). Since nucleic acid-based assays are generally much more sensitive than ELISA-based assays for antigens, the observed sensitivity of Enterichek for the detection of BRV-A or BCoV in feces may be acceptable as an animal-side test if the test is performed on samples collected from calves at acute stage when a large number (i.e., ≥10^3 virus particles) of rotaviruses and/or coronaviruses are expected to shed. However, such poor specificity of Enterichek for BCoV was unexpected. The specificity of the antibodies used in the test for capturing and/or detecting BCoV may need to be re-evaluated or modified. Until such time, positive results of the kit for BCoV should be interpreted with caution and the sample may need to be submitted to a diagnostic laboratory for confirmation.

Several technical concerns with the kit were identified while testing the fecal samples in this study. First, the dilution tube provided with the kit has narrow opening; therefore, the tube can hold only one strip at a time (Fig. 2B) if one follows the
manufacturer’s recommendation to keep strips separated while in the dilution tube. This means it would take at least 40 minutes to compete the testing for 4 pathogens (i.e., 10 min with each strip). In the current study, a modification had to be made to accommodate all 4 strips in a fecal suspension at the same time to save testing time (Fig. 2A). Second, the strips for different targets varied in absorption/migration rate even though testing was done on the same sample (Fig. 3). Hence, running time could vary between strips. Third and importantly, clogging occurred in strips when semi-solid samples were tested so that testing could not be done within the timeframe (~ 10 min) as directed by the manufacturer or even with extended time (i.e., > 20 min). Solving this problem requires further dilution (e.g., 2X or 4X) of samples, but such a protocol modification would lower the sensitivity of the kit. These technical drawbacks should be taken into consideration when using the kit in the field.

In conclusion, this kit should be applied to samples collected from acutely affected animals. Sample pooling may not be recommended. The kit can be a rapid animal-side test tool for C. parvum and E. coli K99+ in the field. However, care must be taken when interpreting BCoV positive and BRV-A negative results of the kit and the sample may need to be submitted to a veterinary diagnostic laboratory for further testing.

ACKNOWLEDGEMENT

The authors would like to thank the Jessica Boor and Jacqueline Thomas for their excellent assistance in sample collection from VDL submissions as well as Dr. Rodger
Main, Director of ISUVDL, for his understanding and being supportive of research. The study was supported in part by funding from ISUVDL Research and Development Fund, Iowa Beef Center, and USDA CSREES (Award No. 2007-35102-18115).

**SOURCES AND MANUFACTURERS**

a. Bovine Enterichek, Biovet®, Saint-Hyacinthe, Quebec, Canada  
b. MagMax™ Total Nucleic Acid Isolation Kit, Applied Biosystems, Austin, TX, USA  
c. AgPath-ID™ Multiplex RT-PCR kit, Applied Biosystems, Austin, TX, USA  
d. ABI 7500 Fast Real-Time PCR System, Applied Biosystems, Austin, TX, USA  

**REFERENCES**


Table 1. Comparative performance of the Bovine Enterichek kit and bovine enteric panel in detecting bovine coronavirus (BCoV), bovine rotavirus A (BRV-A), *Escherichia coli* (*E. coli*) K99⁺, or *Cryptosporidium parvum* (*C. parvum*) from fecal samples.

<table>
<thead>
<tr>
<th>Enterichek</th>
<th>PCR</th>
<th>Total</th>
<th>% agreement (κ value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Entericheck</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>BCoV</td>
<td>Positive</td>
<td>18</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>12</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>BRV-A</td>
<td>Positive</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>15</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>Total</td>
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<td>74</td>
</tr>
<tr>
<td><em>E. coli</em> K99⁺</td>
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<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
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<td>93</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>7</td>
<td>93</td>
</tr>
<tr>
<td><em>C. parvum</em></td>
<td>Positive</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>5</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>27</td>
<td>73</td>
</tr>
</tbody>
</table>
Figure 1. Components of Bovine Enterichek kit.
Figure 2. Photography of test set-up using Bovine Enterichek kit. Panel A shows the modified set-up to use all 4 strips of the kit simultaneously for detection of bovine coronavirus, bovine rotavirus A, *Escherichia coli K99*<sup>+</sup> and *Cryptosporidium parvum* in a fecal sample. The strips are hung together by a paper clip and immersed into a container with the diluted sample. Panel B shows how to run the test using a dilution tube provided in the kit as per manufacturer’s recommendation.
**Figure 3.** Different migration rates among strips of Bovine Enterichek kit. Each of 4 strips targets different pathogen (bovine coronavirus, bovine rotavirus A, *Escherichia coli* K99\(^+\) or *Cryptosporidium parvum*) and they were used simultaneously to test the same sample. The strips were kept immersed approximately 10 min before photographed.
CHAPTER 4. DETECTION AND MOLECULAR CHARACTERIZATION OF BOVINE NOROVIRUS AMONG BOVINE DIARRHEA CASES IN THE MIDWEST USA

A paper to be submitted to Journal of Clinical Microbiology

Yong-II Cho, Jae-Ik Han, Dong Sun, Sang-Ik Park, Vickie Cooper, Kent Schwartz, Kyoung-Jin Yoon

ABSTRACT

Bovine norovirus (BNoV) is a member of genus Norovirus, family Caliciviridae. While the role of BNoV in enteric disease remains unclear, norovirus is a major cause of non-bacterial acute gastroenteritis in human. To characterize contemporary BNoV in bovine populations, fecal samples (n=102) from clinically diarrheic animals were obtained from laboratory submissions during 2010. The samples were from 82 different cattle herds in 8 states and were tested by a real-time RT-PCR (rRT-PCR). BNoV was detected in 53 samples (52%), suggesting endemic status in diarrheic bovine and emphasizing the need for further evaluation of its clinical significance. Among 38 BNoVs successfully sequenced for polymerase gene, 14 and 24 BNoVs were phylogenetically classified into GIII-1 and GIII-2, respectively. Interestingly GIII-1 BNoVs were identified at a much higher rate than expected based on previous reports in US, the significance of which requires a further study. Sequence divergence between the 2 genotypes was 18.6-24.3%,
while sequence identity within each genotype was 82-100% (GIII-1) and 85.8-100% (GIII-2), respectively. Clustering with ≥10% sequence divergence between clusters was observed within each genotype, justifying establishment of subtypes. Besides mutations, recombination among BNoVs appeared to occur frequently since the genotype of viruses was frequently switched when compared by capsid gene, raising the need for better classification criteria. This study found BNoV widely distributed among diarrheic bovine in the Midwest USA with considerable genetic diversity.

INTRODUCTION

Bovine norovirus (BNoV), belonging to genus *Norovirus* in the family *Caliciviridae*, is non-enveloped, single-stranded positive-sense RNA virus with small genome of 7.4 -8.3kb in size (2, 9). The viral RNA genome comprises 3 open-reading frames (ORFs). ORF1 encodes the polyprotein that is cleaved into 6 nonstructural proteins, one of which is viral RNA-dependent RNA polymerase (RdRp). ORFs 2 and 3 encode the major and minor capsid proteins (VP1 and VP2), respectively (5). Due to a high level of genetic diversity among noroviruses (NoVs), 5 genogroups (G) have been identified based on entire ORFs 2 and 3 sequences (30). BNoV belongs to GIII genogroup that has two prototype strains, Jena (genotype 1; GIII-1) and Newbury 2 (genotype 2; GIII-2) viruses and is phylogenetically distinct from human (GI, GII and GIV), porcine (GII-11, GII-18 and GII-19) and murine (GV) NoVs (11, 15, 22).

Noroviruses are a major cause of non-bacterial acute gastroenteritis in humans (10). Noroviruses are also known to cause gastroenteric disease in animals such as cattle, pigs,
dogs and mink (22). In newborn calves experimentally inoculated with Jena strain of BNoV via oral route, the virus infected epithelial cells of small intestine, caused villous atrophy (jejunum and ileum) and diarrhea with shedding, but induced no seroconversion (18). Detection of BNoV in feces from clinically healthy bovine, however, has also been reported (7, 14, 21).

The possibility of interspecies transmission of NoV was proven by a study demonstrating infection of gnotobiotic pigs by a human NoV strain, raising a concern for its zoonotic potential world-wide (11, 20). Numerous studies have been recently conducted to survey BNoV infection in cattle and to molecularly characterize the viruses in comparison to human NoVs. The reported frequency of BNoV detection as measured by molecular methods widely varied among different countries, ranging from 7.5% to 49.6% (4, 7, 8, 12, 17, 19, 21, 23, 25, 29). All identified BNoVs were phylogenetically distinct from human NoVs, suggesting that zoonotic potential of BNoV is unlikely.

In the US, two surveys for BNoV were conducted on veal calves (n=111) in two farms in Ohio, diarrheic neonatal calves (n=62) in eight dairy farms in Michigan, and feces (n=14) collected from 14 different dairy farms in Wisconsin between 2000 and 2003, suggesting 70-80% prevalence of BNoV in the studied populations (23, 27). Since then, there are no recent reports of BNoV prevalence in cattle populations covering wide geographic distribution in the US or molecular characterization of more contemporary viruses circulating in the field. The following study was conducted to assess the frequency of detection and genetic relatedness of BNoV among diarrheic animals from cattle herds in 8 different states during 2010.
MATERIALS AND METHODS

Specimens. One-hundred-two bovine feces were used for the study. The samples were procured from submissions from diarrheic calves to the Veterinary Diagnostic Laboratory at Iowa State University in 2010. In addition, fecal samples from 4 pigs and 1 goat were included as negative controls for PCR to assess any cross-species transmission of BNoV. Sixty-one percent of the bovine samples came from calves less than 1 month of age, 31% from calves at between 1 and 6 months of age, and 8% from calves older than 6 months of age. The samples were from 82 different cattle farms with the majority of the samples (99%) originated in the Midwest USA [Iowa (58%), Minnesota (15%), Wisconsin (7%), Missouri (6%), Ohio (5%), Illinois (3%), South Dakota (3%) and Nebraska (2%)]. The remaining samples (1%) were from a cattle farm in Florida. No more than 3 samples were collected from the same farm. Fifty-seven percent and 25% of the samples derived from dairy and beef breeds, respectively. The remaining 18% of the samples were submitted without breed identification.

Polymerase chain reaction (PCR) assays. A previously reported real-time reverse transcriptase (rRT)-PCR (28) was used to detect BNoV in fecal samples. The PCR target was junction sequence between ORF1 and ORF2 of the virus. First, each fecal sample was suspended in 0.01M phosphate-buffered saline (pH 7.4) to make 30% fecal homogenates and then centrifuged for 1 min at 100 × g for clarification as previously described(1). The supernatant was then used for viral RNA extraction using MagMax™ Total Nucleic Acid Isolation Kit (Applied Biosystems, Austin, TX) according to the manufacturer’s instruction.
The extraction procedure was performed by using Kingfisher® 96 Magnetic Particle Processor (Thermo Fisher Scientific Inc., Waltham, MA). All extracts were stored at -80°C until tested.

PCR was performed with AgPath-ID™ One-Step RT-PCR Kit (Applied Biosystems, Austin, TX) as previously described (1). In brief, 5 µl of extract was mixed with 20 µl of the reaction mixture containing 400 nM of each primer (Table 1), 120 nM of the probe (Table 1), RT-PCR buffer, RT-PCR enzyme mix, and nuclease-free water. Amplification of the targeted genomic region was performed using ABI 7500 Fast Real-Time PCR System. The cycling condition were as follows: RT for 10 min at 45°C and 15 min activation for the DNA polymerase at 95°C, followed by 40 cycles of denaturation at 94°C for 10 sec and annealing/extension at 60°C for 60 sec. Samples with cycle threshold (Ct) of ≤ 40 were considered positive for BNoV.

**Sequencing of BNoV RdRp and capsid genes.** Real-time RT-PCR- positive samples were subjected to 2 conventional RT-PCR assays to amplify partial RdRp gene (326 bp) or partial VP1 gene (512 bp) of BNoV as previously described (19, 23, 28) with specific primer sets for each target gene as described in Table 1.

For the amplification of the RdRp gene region, both RT-PCR and nest PCR (19, 23) were conducted using OneStep RT-PCR Kit (QIAGEN, Valencia, CA) with QIAGEN® RNase inhibitor and HotStarTaq® DNA Polymerase Kit (QIAGEN, Valencia, CA), respectively, according to the manufacturer’s instruction. Cycling conditions of the RT-
PCR were: reverse transcription at 50°C for 30 min; activation of DNA polymerase at 95°C for 15 min; 40 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 1 min; and followed by a final cycle at 72°C for 7 min. Cycling conditions of the nested PCR were: activation of DNA polymerase at 95°C for 15 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min; and followed by a final cycle at 72°C for 10 min.

For the amplification of the VP1 gene region, a conventional RT-PCR (28) was performed using OneStep RT-PCR Kit (QIAGEN, Valencia, CA) with QIAFAST® RNase inhibitor. Cycling conditions were: reverse transcription at 50°C for 30 min; activation of DNA polymerase at 95°C for 15 min; 40 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min; and followed by a final cycle at 72°C for 10 min. All amplified products were visualized by electrophoresis in 1.5% agarose gel with SYBR® Safe DNA gel stain (Invitrogen, Eugene, Oregon).

PCR products were purified with QIAquick® purification kits (QIAGEN, Germantown, MD) by following the procedures recommended by the manufacturer. The purified PCR products were submitted to the ISU Nucleic Acid Facility for sequencing. Sequencing was repeated once to ensure the fidelity of sequence data. Once sequence data were available, sequence alignments and phylogenetic analyses were carried out using Lasergene® software (DNASTAR Inc., Madison, WI, USA). Phylogenetic trees were generated using MEGA4 software (24) based on neighbor-joining method with a 1,000 bootstrap replicates. The reference BNoV strains used in the sequence comparison were: AB074892, AF093797, AF097917, AF414427, AF542083, AF542084, AJ011099,
RESULTS

Prevalence of BNoV in clinical cases. Among the 102 fecal samples tested, 53 samples (52%) were positive for BNoV nucleic acid representing 41 of the 82 farms (50%), while the feces from the other 4 porcine and 1 caprine were negative for BNoV. In terms of age distribution, 73.6% of the 53 BNoV-positive samples were from calves less 1 month of age (n=39), 22.6% from calves between 1 and 6 months of age (n=12) and only 3.8% from calves older than 6 months of age (n=2).

Phylogenetic analysis based on partial RdRp sequence. Among the 53 samples positive by rRT-PCR for BNoV, a 326-nucleotide segment of the RdRp gene, which was closer to its 3’ terminus, was successfully amplified and sequenced from 38 samples (Table 2). When compared to the corresponding sequence of the selected reference strains, all of the 38BNoVs phylogenetically belonged to genogroup GIII (Fig. 1). Fourteen of those viruses were clustered within genotype GIII-1 represented by “Jena” strain (9) identified as AJ011099 in Figure 1, while the remaining 24 viruses were classified to genotype GIII-2 represented by “Newbury 2” strain (3) identified as AF097917 in Figure 1. Interestingly, 10 of the 24 GIII-2 BNoVs were phylogenetically out-branched (tentatively designated as GIII-2b) from the remaining 14 BNoVs and previously reported GIII-2
BNoVs (tentatively designated as GIII-2a) and were further divided into two clades (tentatively designated as GIII-2b₁ and GIII-2b₂), while GIII-2a BNoVs could be subdivided into four different clades. Similarly, GIII-1BNoVs could also be divided into 2 subtypes (tentatively designated as GIII-1a and GIII-1b). The majority of the viruses were clustered in GIII-1b which included “Jena” strain, prototype GIII-1 BNoV.

Sequence identities between GIII-1 and GIII-2 BNoVs were 75.7-81.4 %, while identities among BNoVs within each genotype were 82.0-100% (GIII-1) and 85.8-100% (GIII-2), respectively. GIII-2b BNoVs shared 86.4-89.9% and 77.2-83.8% sequence homologies with GIII-2a and GIII-1 BNoVs, respectively. GIII-2b₁ and GIII-2b₂ BNoVs shared 85.5-91.5% homologies between the subtypes, while BNoVs in each subtype showed 92.1-93.7% (GIII-2b₁) and 89.3-98.7% (GIII-2b₂) sequence identities among them respectively. Regarding GIII-1 BNoVs, viruses in each subtype had sequence homologies of 87.1-91.7% (GIII-1a) and 79.2-100% (GIII-1b) respectively, while sequence divergence of 17.0-22.9% was observed between the two subtypes.

**Phylogenetic analysis based on partial VP1 gene.** Among the 53 samples positive for BNoV by rRT-PCR, 30 samples were positive for the VP1 gene of BNoV by the conventional RT-PCR. Sequencing of PCR products (520bp) was successful on 14 of the 30 samples (Table 3). In comparison to the reference strains, 3 BNoVs were clustered with NoVs belonging to GIII-1 genotype and the remaining 11 BNoVs were closely related to GIII-2 NoVs (Fig. 2). Sequence homologies within each genotype were 85.0-99.6% (GIII-1) and 85.8-100% (GIII-2), respectively. The 11 GIII-2 BNoVs were subdivided into 2
clusters (tentatively designated as GIII-2A and GIII-2B) which showed 10.9-16.9% sequence divergence between them.

Interestingly, 5 (Bo/Nov-8, 19, 20, 28 and 35) of the 11 GIII-2 BNoVs were classified as GIII-1 when they were analyzed based on their RdRp gene sequences, while 2 (Bo/Nov-42 and 44) and 6 strains (Bo/Nov-13, 36, 37, 41, 45 and 46) were clustered with GIII-1 and GIII-2, respectively, based on sequences of both genes. It could not be determined whether or not genotype classification of Bo/Nov-22 was same or different depending upon the gene to compare because sequencing of its RdRp gene failed. Sequence identities of Bo/Nov-22 with GIII-1 and GIII-2 BNoVs for the VP1 gene target were 76.3-78.3% and 75.9-78.9%, respectively, suggesting that the virus is likely a distinct strain of BNoV which does not belong to any of the known genotypes (i.e., GIII-1 or GIII-2).

**DISCUSSION**

The detection rate of BNoV infection reported from different countries and regions varies considerably (4, 7, 8, 12, 17, 19, 21, 23, 25, 29): 20.8% in Italy (2011), 20% in France (2011), 8.5% in Turkey (2011), 49.6% in Norway (2010), 7.5% in Belgium (2009), 8.5% in Hungary (2009), 9.3% in Korea (2007), 8.0% in England (2003), 8.9% in Germany (2003); 70-80% in US (2002-2003), and 31.6% in the Netherlands (2003). In this study, the frequency of BNoV detection in feces from diarrheic bovine submitted to the Iowa State University Veterinary Diagnostic Laboratory was 52%. Large variation of
reported detection rate among countries/regions could be attributed to: 1) regional variation of BNoV infection due to populations, density or management factors; 2) difference in ages of animals examined (e.g., calves versus adult); 3) application of different sampling method (e.g., one or two large size herd versus random sampling); 4) clinical status of animals sampled (e.g., feces from clinical versus asymptomatic bovine); and 5) variation in detection method used (e.g., PCR for virus versus ELISA for antibody) or test platform for agent detection (e.g., real-time PCR, nested RT-PCR or RT-PCR).

Even though the frequency of BNoV status in this study was lower than that previously reported in the US (23, 27), our data still suggest that BNoV is common in diarrheic animals. The frequency of detection in this study is likely representative of status of BNoV infection in the studied populations because: a) The fecal samples examined in the study were originated in wide geographical regions in the USA (i.e., Iowa, Minnesota, Wisconsin, Nebraska, South Dakota, Missouri, Illinois and Florida); b) The samples represented a large number of farms (n=82); and c) The samples were from both dairy and beef cattle.

The common presence of BNoV in diarrhea calves raises the need for further evaluation of its clinical significance in the field. Although one or more of other bovine enteric pathogens (e.g., bovine rotavirus, bovine coronavirus, bovine nebovirus, bovine torovirus, bovine viral diarrhea virus, Salmonella spp, Escherichia coli K99+, Cryptosporidium parvum) were concurrently detected with BNoV in many of the samples examined in the study (data not shown), BNoV was solely detected in 6% of the tested samples. This suggests that BNoV may have a role as a pathogen or co-pathogen similar to other endemic agents (e.g., coronavirus, rotavirus, cryptosporidia) that are known to be
common contributors to calf scouring. A recent animal study reported that a GIII-1 BNoV (Jena strain) was able to cause intestinal lesions and diarrhea in newborn calves under experimental conditions when given orally (18). Nevertheless, a case-control study or cohort longitudinal study and animal challenge study with contemporary strain may be necessary to further assess the clinical significance of BNoV in calf diarrhea since only sick animals were the study population for the present study and shedding of BNoV from clinically healthy animals has been reported (7, 14, 21).

It is known that a high degree of genetic diversity exists among NoVs (14). Two studies reported increase in the number of identifiable genetic clusters (i.e., genotypes) among 5 genogroups from 29 to 31 within 1 year due to rapid genetic changes (26, 30). In agreement with previous reports, our study also revealed a high genetic diversity among BNoVs detected in diarrheic calves. Several notable observations were made in sequence analysis. First, all of BNoVs identified and sequenced in the study were classified into GIII, which is in agreement with previous studies (4, 7, 8, 12, 13, 17, 19, 21, 27, 29) and also diminishes public health concern of zoonotic BNoV since all NoVs identified in humans to date belong to GI, GII or GIV. Second, any particular genotype or cluster was not strongly associated with geographic origin of the studied herds. Third, GIII-1 BNoV was identified at a much higher rate (39%) than what was previously reported in US based on RdRp gene sequence (23, 27), even though GIII-2 BNoV was still the dominant genotype in the animals examined in this study. Unfortunately the biological significance of higher detection of GIII-1 BNoV could not be assessed in the study. Fourth, some of the GIII-2 BNoVs identified in the study showed >10% divergence of RdRp sequences from the previously reported GIII-2 BNoVs. Since the region (326 bases) of RdRp gene
used for sequence analysis has been reported to be relatively conserved among NoVs (28), more than 10% sequence difference in a conserved gene is significant, justifying establishment of subdivisions (e.g., clade, cluster or subtype) (30) within genotype GIII-2 as we proposed in the study, i.e., GIII-2a and GIII-2b (Fig. 1) or GIII-2A and GIII-2B (Fig. 2). A wide range of nucleotide homologies among GIII-1 NoVs (82.0-100%) also support establishment of subtype/clade classification for GIII-1 NoVs, for example GIII-1a and GIII-1b (Fig. 1).

Although the RdRp gene of NoV has been commonly used for molecular epidemiology and phylogenetic analysis, some investigators suggested that the VP1 gene is more suitable for the same purpose (27, 30). Assessment of potential antigenic relatedness among viruses would be additional merit since NoV is a non-enveloped virus and capsid protein is the major structural protein of the virus. In our study, viruses were compared using both RdRp and VP1 gene sequences. Under conditions present in the study, sequencing for the capsid gene was less successful than that for RdRp gene, presumably due to a higher genetic variability of the capsid gene, although suboptimal PCR and sequencing conditions could account for it. Based on partial sequence of the VP1 gene, most (80%) of the sequenced BNoVs were clustered with GIII-2 NoVs, whereas GIII-1 was a more common genotype in previous reports (27). More importantly, it was noted that some of BNoVs were classified into a different genotype depending upon which gene was used for phylogenetic analysis. This observation supports the occurrence of genetic recombination among NoVs as previously reported (6, 13, 16), although the possibility that two different genotypes of NoVs were present in the same samples cannot be completely ruled out because sequencing was done directly on the samples and virus isolation
followed by plaque-cloning or limiting dilution could not be employed. The observation that 45% of the GIII-2 BNoV (based on the VP1 gene) were shifted to GIII-1 (based on the RdRp gene) in their classification suggests that recombination among NoVs might occur at a higher rate than what was previously believed. In this sense, use of both RdRp and VP1 gene sequences for molecular epidemiology would be necessary. It is interesting to note that those recombinant BNoVs (e.g., Bo/NoV-8, 19, 20, and 28) phylogenetically tended to be clustered closely (e.g., GIII-2B in Figure 2) and related to the previously reported recombinant BNoVs (FM242195, FM232196, FM242198, AY126468), suggesting that recombinant BNoVs had gone through molecular evolution independent from other BNoVs and may have formed a new group (7).

ACKNOWLEDGEMENT

The authors would like to thank Jessica Boor and Jacqueline Thomas for their excellent assistance in sample collection from VDL submissions. The study was supported in part by funding from ISUVDL Research and Development Fund, Iowa Beef Center, USDA CSREES (Award No. 2007-35102-18115), and Korean MFAFF National Veterinary Research and Quarantine Service (Award No. C-AD14-2006-10-2).
REFERENCES


TABLE 1. Primers and probe used for bovine norovirus (BNoV)-specific PCR assays in the study

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**TABLE 2.** Name, GenBank accession numbers and origin of state of the BNoV strains used in RNA-dependent RNA polymerase gene phylogenetic analysis

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FIG. 1. Phylogenetic relationship between newly identified bovine noroviruses (BNoVs) in the US Midwest cattle population and previously reported BNoVs and NoVs based on the partial nucleotide sequence of RNA-dependent RNA polymerase. The trees were constructed using the neighbor-joining method of MEGA4. The newly identified BNoVs are described with given name and their GenBank accession numbers in Table 2.
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CHAPTER 5. A CASE-CONTROL STUDY OF MICROBIOLOGICAL ETIOLOGY ASSOCIATED WITH CALF DIARRHEA

A paper to be submitted to Veterinary Microbiology

Yong-Il Cho, Jae-Ik Han, Chong Wang, Vickie Cooper, Kent Schwartz, Terry Engelken, Kyoung-Jin Yoon

ABSTRACT

Calf diarrhea is a major economic burden for the US cattle industry. A variety of infectious agents are implicated in calf diarrhea and co-infection of multiple pathogens is not uncommon in diarrheic calves. A case-control study was conducted to assess infectious etiologies associated with calf diarrhea in Midwest cattle farms. A total of 199 and 245 fecal samples were obtained from diarrheic and healthy calves, respectively, from 165 cattle farms. Samples were tested by a panel of multiplex PCR assays for 11 enteric pathogens: bovine rotavirus group A (BRV-A), bovine coronavirus (BCoV), bovine viral diarrhea virus (BVDV), bovine enterovirus (BEV), bovine norovirus (BNoV), Nebovirus, bovine torovirus (BToV) Salmonella spp. (Salmonella), Escherichia coli (E. coli) K99+, Clostridium perfringens (C. perfringens) with β toxin gene and Cryptosporidium parvum (C. parvum). The association between diarrhea and detection of each pathogen was analyzed using a multivariate logistic regression model. More than a half of the fecal samples from the diarrheic calves had multiple pathogens. Statistically, BRV-A, BCoV,
BNoV, Nebovirus, *Salmonella*, *E. coli* K99\(^+\), and *C. parvum* were significantly associated with calf diarrhea (*p*<0.05). Among them, *C. parvum* and BRV-A were considered to be the most common enteric pathogens for calf diarrhea with high detection frequency (33.7% and 27.1%) and strong odds ratio (173 and 79.9). Unexpectedly BNoV (OR=2.0) and Nebovirus (OR=16.7) were identified with high frequency in diarrheic calves, suggesting these viruses may have a significant contribution to calf diarrhea.

**INTRODUCTION**

Calf diarrhea is a major cause of economic loss with high morbidity and mortality in the cattle industry worldwide (3, 12, 29, 57, 58). Many factors are known to contribute to calf diarrhea. Historically, calf diarrhea has been commonly attributed to bovine rotavirus group A (BRV-A), bovine coronavirus (BCoV), bovine viral diarrhea virus (BVDV), *Salmonella* spp. (*Salmonella*), *Escherichia coli* (*E. coli*) K99\(^+\), and *Clostridium perfringens* (*C. perfringens*) type C and *Cryptosporidium parvum* (*C. parvum*) (1, 49, 50, 52). The specific etiology of many field cases of calf diarrhea still remain undiagnosed (39). Recently, bovine norovirus (BNoV), Nebovirus, bovine enterovirus (BEV) and bovine torovirus (BToV) have been identified as potential causes of calf diarrhea (5, 20, 21, 28, 43-46). Some of these agents (i.e., BNoV, BEV and BToV) have also been found in feces from clinically healthy calves (20, 25, 38, 51) and many of previous epidemiological studies for BNoV and BToV have been focused only on diarrheic calves (22, 39, 44, 46). Their role in calf diarrhea still remains to be evaluated.

Various laboratory methods have been applied for the detection of infectious agents in feces. Historically, virus isolation, electron microscopy, enzyme-linked immunosorbent
assay, latex agglutination test, bacterial culture, direct microscopy of fecal smear (acid-fast stain), and/or fecal flotation have been commonly used to test fecal samples for enteric pathogens (16, 37). These procedures are reliable; however they are time-consuming and require specialized knowledge. Recently, nucleic acid based tests, such as polymerase chain reaction (PCR) assays, have become popular for rapid and sensitive detection of infectious agents (2, 9). Multiplex real-time PCR panels have been proven to be a useful diagnostic tool for concurrent detection of several target enteric pathogens with high sensitivity and specificity (2, 9), which decreases bias in diagnostic outcome due to testing method.

The following case-control study was conducted to: a) assess the prevalence of 11 infectious agents consisting of 7 common [BRV-A, BCoV, BVDV, Salmonella, E. coli K99+, C. perfringens with β toxin gene (Cpt β) and C. parvum] and 4 emerging enteric pathogens (BNoV, Nebovirus, BEV and BToV) in fecal samples from healthy and diarrheic calves in the Midwest by using a multiplex real time PCR panel; and b) determine their association with diarrhea as well as investigate their potential interactions in expression of disease.

MATERIALS AND METHODS

Animals and samples. All fecal samples used in the study were originated from clinically diarrheic and healthy calves during year 2010-2011. A total of 199 fecal samples from diarrheic calves were procured from submissions to the Iowa State University Veterinary Diagnostic Laboratory (ISUVDL) and used as cases. The samples were from 140 cattle farms with the most of the samples (99%) originated in the Midwest [Iowa
(78%), Minnesota (8%), Wisconsin (4%), Missouri (3%), Ohio (3%), Illinois (1%), South Dakota (1%) and Nebraska (1%)]. No more than 4 samples were selected from the same farm. Approximately 41% and 42% of the samples were from dairy and beef breeds, respectively. The remaining 18.5% of the samples were submitted without breed identification. Physical appearance of first 99 of the 199 fecal samples was recorded as ‘watery’ or ‘semi-solid’ upon receiving as fresh samples were available to the investigators before freezing.

A total of 245 fecal samples were collected from clinically healthy calves in 25 different beef or dairy farms which were evenly distributed across the State of Iowa and used as controls. Samples were collected twice from each farm at approximately 2-week intervals with continuous monitoring of health status including lack of diarrhea. At each time of sample collection, 5 calves were randomly selected for sampling.

Most (96.4%) of the calves tested were less than 6 months old in age. Only 1 and 7 cases were submitted from a 7-month-old diarrheic calf and clinically healthy yearlings or older cattle, respectively.

**Detection of pathogens.** All fecal samples were examined for 11 different microorganisms (i.e., BRV-A, BCoV, BVDV, BEV, BNoV, BToV, Nebovirus, *Salmonella*, *E. coli* K99+, *C. parvum* and Cpt β) using a panel of polymerase chain reaction (PCR) based assays. All except BEV have been reported as pathogens implicated in calf diarrhea. Before PCR testing, each fecal sample was suspended in 0.01M phosphate-buffered saline (pH 7.4) to make 30% fecal homogenates and then centrifuged for 1 min at 100 × g for clarification as previously described (9). The supernatant was then used for viral and
bacterial nucleic acid extraction using MagMax™ Total Nucleic Acid Isolation Kit (Applied Biosystems, Austin, TX) according to the manufacturer’s instruction. The extraction procedure was performed using Kingfisher® 96 Magnetic Particle Processor (Thermo Fisher Scientific Inc., Waltham, MA). All extracts were stored at -80°C until tested.

Probe-based real-time PCR (rtPCR) assays for all pathogens except BToV and Nebovirus were performed in a duplex or singleplex PCR format with Path-ID™ Multiplex One-Step RT-PCR Kit (Applied Biosystems, Austin, TX) and AgPath-ID™ One-Step RT-PCR Kit (Applied Biosystems, Austin, TX), respectively. For BToV, a SYBR Green rtPCR assay was used with QuantiTest™ SYBR® Green PCR Kit (QIAGEN, Valencia, CA).

For rtPCR set-up, 7µl of template and 18 µl of the reaction mixture for the duplex PCRs (Table 1, real-time PCR set 1, 2, 5 and 6) and 5µl of template and 20 µl of the reaction mixture for singleplex PCRs (Table 1, real-time PCR set 3 and 4) were used. All reaction mixtures contained 400 nM of each primer, 120 nM of the probe except BToV, RT-PCR buffer, RT-PCR enzyme mix, and nuclease-free water. The volume of each reagent added to a reaction mixture was as per manufacturer’s instruction. The sequence information of primers and probes used for specific detection of each pathogen is summarized in Table 1.

Amplification of the targeted genomic region was conducted using ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Austin, TX). Cycling conditions of the probe-based rtPCRs were as follows: a) reverse transcription (RT) for 10 min at 48°C (45°C for singleplex); b) activation of DNA polymerase at 95°C for 15 min (10 min for
singleplex); and c) 40 cycles of denaturation at 94°C for 10 sec and annealing/extension at 60°C for 60 sec (45 sec for singleplex). The RT step was applied only for viral targets. Running conditions of the SYBR Green rtPCR for BToV were: a) RT step for 10 min at 50°C; and b) 40 cycles of denaturation at 95°C and annealing/extension at 60°C for 30 sec. After 40 cycle reaction, the melting curve analysis was performed. Samples with cycle threshold (Ct) ≤ 35 for any given targets were considered positive for those pathogens.

For detection of Nebovirus, a gel-based nested RT-PCR was used as previously described (27). The PCR was conducted using OneStep RT-PCR Kit (QIAGEN, Valencia, CA) with QIAGEN® RNase inhibitor and HotStarTaq® DNA Polymerase Kit (QIAGEN, Valencia, CA) for RT-PCR and nested PCR, respectively, according to the manufacturer’s instruction. Cycling conditions of the RT-PCR were: a) RT step at 50°C for 30 min; b) DNA polymerase activation step at 95°C for 15 min; c) 40 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 1 min; and d) followed by a final cycle at 72°C for 10 min. Cycling conditions of the nested PCR were: a) activation step at 95°C for 15 min; and b) 35 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec and extension at 72°C for 1 min; and c) followed by a final cycle at 72°C for 10 min.

**Effect of test methods on detection frequency of enteric pathogens.** Detection frequencies of selected enteric pathogens (i.e., BRV-A, BCoV and *C. parvum*) in calf diarrhea cases submitted to ISUVDL from 2003 to 2011 were compared based on laboratory methods used. Before 2008, antigen-capturing ELISA and fecal smear direct microscopy (acid-fast stain) tests were used to detect BRV-A/BCoV and *C. parvum* in
feces, respectively. Since then, a bovine enteric panel consisting of 2 multiplex rtPCR tests (9) was implemented for simultaneous detection of BRV-A, BCoV, and *C. parvum* in feces. All diagnostic data were retrieved from the ISUVDL laboratory information management system.

**Statistics.** The PCR results on each of the fecal samples were recorded as either positive or negative for each pathogen and categorized under disease status (i.e., diarrheic versus non-diarrheic) of each animal. The association between diarrhea and detection of each pathogen was determined using a multivariate logistic regression model. The probability of concurrent detection among pathogens was also analyzed in the same manner. The final model was built with stepwise selection using Firth’s penalized likelihood method due to quasi-complete separation of the data. Odds ratios (ORs) with 95% confidence intervals were calculated to assess the likelihood of association.

The association between the severity of diarrhea (i.e., watery versus semi-solid) and the presence of each pathogen was also analyzed using multivariate logistic regression model with stepwise model selection.

Since BNoV and BCoV were detected in feces from both diarrheic and healthy calves at a relatively high frequency, Ct values of feces for BNoV and BCoV were analyzed by the non-parametric Wilcoxon rank-sum test to evaluate the quantitative difference in virus shedding between diarrheic and healthy calves.

All statistical analyses were conducted by using SAS 9.2 (SAS Institute, Cary, NC). For all analyses, a value of *p* <0.05 was considered significant.
RESULTS

Survey of calves in the Midwest USA for infection with enteric pathogens. A total of
the 199 fecal samples from diarrheic calves and 245 fecal samples from healthy calves
were tested for 11 putative enteric pathogens. PCR testing revealed that 80.4% and 27.8%
of the diarrheic and normal fecal samples, respectively, were positive for at least one of
these infectious agents.

As summarized in Table 2, BNoV (44.7%), C. parvum (33.7%), BCoV (31.7%),
BRV-A (27.1%), Nebovirus (21.6%) and Salmonella (9.0%) were commonly detected in
feces from the diarrheic calves, while BVDV, BToV, E. coli K99+ and BEV were found at
a much lower frequency (0.4 to 5%). BNoV (16.3%) and BCoV (12.2%) were also
detected in the feces from healthy calves but at a lower frequency than that in diarrheic
feces. While Nebovirus (1.6%) and BVDV (0.4%) were infrequently detected in the feces
from healthy calves, C. parvum, BRV-A, E. coli K99+ and BToV were detected only in the
feces from diarrheic calves. In contrast, BEV (32.7%) was much more frequently detected
in the feces from healthy calves than those from diarrheic calves. Clostridium perfringens
with β toxin gene (i.e., C. perfringens type B or C) was not detected in any of the feces
examined in this study.

Although BNoV and BCoV were detected in feces from both diarrheic and healthy
calves, the detection frequency and fecal shedding quantity of the viruses were
significantly higher in the feces from diarrheic calves except for one healthy calf feces
which showed the lowest Ct value (17.4) for BCoV (Fig. 1), as compared to those in the
feces from healthy calves. The median (mean) Ct values of feces from diarrheic calves
positive for BNoV and BCoV were 26.2 (26.4) and 25.6 (24.3) respectively, whereas those
from healthy calves positive for BNoV and BCoV were 31.3 (31.0) and 31.3 (30.2) respectively.

With respect to age distribution, many of fecal samples from diarrheic calves positive for BNoV, C. parvum, BCoV, BRV-A, Nebovirus, Salmonella, BToV, and E. coli K99⁺ were from calves at 0 to 4 weeks of age (Fig. 2). In particular, calves at 0 to 2 weeks of age were the most commonly positive for these pathogens.

Assessment of the association of 11 enteric pathogens with diarrhea. As summarized in Table 2, the presence of C. parvum, E. coli K99⁺, Salmonella, BRV-A, Nebovirus, BCoV and BNoV in feces were significantly associated with calf diarrhea (p<0.05). Among these pathogens, C. parvum, E. coli K99⁺, Salmonella, BRV-A and Nebovirus showed a stronger association with diarrhea (OR>10.0). In contrast, detection of BEV was inversely correlated with diarrhea (OR=0.113); therefore, BEV was not included in further statistical analyses.

No statistically significant association between the presence of BToV in feces and diarrhea was observed in this study even though the virus was detected only in the feces from diarrheic calves, probably due to a low frequency of detection (Table 2). The ORs could not be calculated for BVDV and Cpt β because of either extremely low frequency of detection or no detection; hence, statistical significance could not be determined.

Bovine rotavirus group A was the only pathogen significantly (p=0.013) associated with liquid form of diarrheic feces (Table 3).
**Concurrent infection of enteric pathogens for calf diarrhea.** While 55% of the diarrheic fecal samples had more than 1 enteric pathogen detected, only 3% of the fecal samples from healthy calves had multiple pathogens (Fig. 3). In the diarrheic fecal samples, the presence of 2 different pathogens (31%) was the most commonly seen and 1% of the samples even had up to 6 different pathogens concurrently.

The probability of detecting certain agents together is summarized in Table 4. Bovine norovirus, BCoV, *Salmonella*, and *C. parvum* were commonly detected in feces which were also positive for BRV-A. Nebovirus was commonly detected in feces also positive for BCoV, *C. parvum* or BToV. BNoV presence was significantly correlated with *C. parvum* presence in addition to BRV-A. While many of the pathogens were concurrently detected with more than 2 other pathogens, BToV and *Salmonella* were identified only with Nebovirus and BRV-A, respectively. The concurrent presence of BToV and Nebovirus was much stronger \(13.4 \leq \text{OR} \leq 15.7\ (2.2-114.5)\] as compared to other mixed infections. Statistically significant synergistic interaction between pathogens for causing the diarrhea or exacerbating the severity of diarrhea was not observed.

When the pathogens were sorted based on their taxonomical property (i.e. virus, bacteria and protozoa) and compared for their detection frequency between diarrheic and healthy calves, virus only (36.2%) or virus/ *C. parvum* co-infection (28.1%) was the most commonly observed in the diarrheic calves. In comparison, virus only (28.0%) was common in the healthy calves (Fig. 4). BNoV and BCoV were the pathogens that were the most commonly detected in the feces from healthy calves.
Influence of laboratory methods on the detection frequency. The mean detection frequency of BRV-A, BCoV and *C. parvum* in diarrhea cases during year 2003-2007 were 24.6%, 11.9% and 8.7%, respectively, when antigen-capturing ELISAs and direct microscopy (acid-fast stain) were for the main laboratory methods for detection of these pathogens at ISUVDL. After implementation of a PCR panel for the major calf diarrhea pathogens, the mean detection frequency of BRV-A, BCoV and *C. parvum* were 37.2%, 29.2% and 38.3%, respectively, during year 2008-2011 (Table 5).

**DISCUSSION**

In this study, we investigated the prevalence of 11 calf enteric pathogens consisting of 7 common (BRV-A, BCoV, BVDV, *Salmonella*, *E. coli* K99+, Cpt β and *C. parvum*) and 4 emerging pathogens (BNoV, Nebovirus, BEV and BToV) and then evaluated two aspects; their clinical significance in calf diarrhea and co-infection between them. Not unexpectedly, 80% of diarrheic calves tested were positive for at least one of the target enteric pathogens, suggesting that the infectious factor is still a major cause of calf diarrhea. More than 50% of the diarrheic calves tested were concurrently infected with more than one pathogen. Co-infection with 2 pathogens was the most common finding (31%) with up to 6 pathogens detected in 1% of the fecal samples from diarrheic calves. The majority of diarrheic cases were identified among 0- to 4-week-old calves and concentrated among calves at 0-2 weeks of age, which is similar to previous reports by other investigators (3, 12, 35). High frequency of co-infection by multiple pathogens in young animals emphasizes that interventions for calf diarrhea should be focused on husbandry and management strategies, including assurance of colostrum intake, hygiene, reduction of
population density, or modified components of the Sandhills calving system (31). Twenty percent of the diarrheic calves were negative for all of the 11 pathogens in this study. While low sensitivity of the test might be accounted for the negative result, the role of non-infectious factors (e.g., cold weather, impaired uptake of colostrum, or poor sanitation) in calf diarrhea cannot be discounted. In addition, the possibility of other pathogens (e.g., rotavirus B or C; coccidia; *clostridium perfringens* type A, D or E; and other pathogroups of *E.coli*) or previously unrecognized agent(s) involved in diarrhea remains to be further studied.

Viral infections (36.1%) or combination of viruses and *C. parvum* (28.1%) were the most commonly detected etiology in feces from diarrheic calves, which is similar to previous reports on calf diarrhea (11, 17, 35). In contrast, the proportion of bacteria-positive samples was relatively small. Of three target bacterial pathogens, *Salmonella* (9%) was the most commonly detected in the diarrhea feces examined. Interestingly, none of the fecal samples from both diarrheic and healthy calves was positive for Cpt $\beta$ which is contained in either *C. perfringens* type B or C. This was an unexpected observation since *C. perfringens* type C has been postulated as the main type causing calf diarrhea.

Although the PCR results were not confirmed by anaerobic bacterial culture, it should be noted that our observation is in agreement with previous reports by other investigators describing no (2, 15, 53) or very low detection of Cpt $\beta$ (18) in diarrheic calves, suggesting that *C. perfringens* type C is rarely involved in outbreaks of calf diarrhea or is simply an opportunistic bacterium causing acute enterotoxemia under certain favorable conditions. As it was suggested that all types of *C. perfringens* should be considered as a calf diarrhea
etiology (15), involvement of other types of *C. perfringens* in diarrhea cases may be necessary.

*Cryptosporidium parvum* was frequently (33.7%) detected in calf diarrhea cases, which is in agreement with previous reports (3, 24, 35, 57). It may imply the difficulty with *C. parvum* control in the field due to autoinfection, environment resistance of oocysts and lack of effective treatment and vaccine (26). Preventative measures for *C. parvum* in cow-calf operations should be focused on keeping good herd sanitation and sick animals segregated from healthy ones (56). Co-infection with viruses (28.1 %), particularly BRV-A (OR=2.7), BNoV (OR=3.6) and Nebovirus (OR=7.1), was much more common than with bacteria in our study. While co-infection of BRV-A and *C. parvum* in diarrheic calves has been frequently reported (3, 4, 12, 17, 57), common association of Nebovirus and *C. parvum* in diarrheic animals is a new observation. It has been reported that viral infections, such as porcine circovirus type 2 and human immunodeficiency virus, can increase the susceptibility of pigs and humans, respectively, to *C. parvum* (41, 47), suggesting that immunosuppressive viruses can predispose animals or humans to *C. parvum*. In the absence of effective treatment options for *C. parvum*, it may be prudent to rely on management practices and specific aids in prevention of viral infections to reduce clinical problems with *C. parvum* infections.

Bovine rotavirus A was found solely in many of the diarrhea cases (27.1 %) and positively correlated with the severity (i.e., liquid feces) of diarrhea (OR=3.3). This observation is similar to reports of human rotavirus infection being highly associated with acute watery diarrhea (42, 61). A high correlation between BRV-A detection and diarrhea (OR=79.9) and a wide range of association with other pathogens (BNoV, BCoV,
Salmonella and C. parvum) may be an evidence that BRV-A is a primary major bovine enteric pathogen of calf diarrhea, which is also in agreement with previous reports describing the primary role of BRV-A in neonatal calf diarrhea (3, 17, 57). Our and others’ observations raise concerns regarding vaccination practices on farms and the efficacy of current licensed BRV-A vaccines since vaccination has been a main tool for prevention of BRV-A associated diarrhea in neonates. Implementation of a regular vaccination program for BRV-A can be easily achieved through enhancing the awareness of the high frequency of rotavirus-associated calf diarrhea in the field, but continuing efficacy of BRV-A vaccines may require frequent surveillance and further characterization of rotaviruses circulating in the field. Surveillance is warranted since antigenic variation of rotavirus due to frequent mutation and recombination is of a great concern for emerging a variant or new serotype (34).

New and emerging viruses with pathogenic potential for calf diarrhea (i.e., BNoV, Nebovirus and BToV) were also studied together with historically well-known major enteric pathogens (i.e., BRV-A, BCoV, BVDV, C. parvum, Salmonella and E. coli K99+ and Cpt β). The most noteworthy observations from our study were the significant association of BNoV (OR=2.0) and Nebovirus (OR=16.7) with calf diarrhea and their frequent detection (44.7% and 21.6%, respectively) in calf diarrhea cases, suggesting that bovine caliciviruses may play a more significant role in calf diarrhea than what was believed. It is an unexpected observation that Nebovirus was detected in diarrheic animals at a much higher rate than what was previously reported from France (28). A high frequency of BNoV detection is, on the other hand, not a surprise since many other investigators have previously reported a high prevalence of BNoV infection in the studied
bovine populations (8, 13, 27, 28, 38, 44, 48, 59, 62, 64). Clinical significance of BNoV infection has not been clear in the field because the virus has also been found in clinically healthy calves (27, 38) as also shown in our study. Recently an animal study has demonstrated that BNoV is pathogenic to naïve calves (43). In our study, which is the first case-control study evaluating BNoV as bovine enteric pathogen for calf diarrhea, a significant quantitative difference in the virus amount between fecal samples from diarrheic and healthy calves was detected, suggesting that disease progression may depend upon the initial exposure dose of the virus or factors contributing to BNoV replication to a high titer. Further study remains to redefine the pathogenicity of bovine caliciviruses and to determine the correlation between virus amount and the pathogenicity and to identify contributing factors.

Unlike bovine caliciviruses, it was difficult to judge the role that BToV may play in calf diarrhea because the virus was detected in a relatively small number of the fecal samples examined (1.1%). Such a detection frequency of BToV in our study is similar to what was previously reported from Korea (2.9%) and Austria (5.2%) but different from that reported in USA (36.4%) and Japan (18%) (14, 20, 30, 46). Although a statistically significant association between BToV and diarrhea could not be demonstrated due to a low prevalence, it must be pointed out that the virus was detected only in feces from diarrheic calves. A survey on a larger number of animals, longitudinal cohort study or animal challenge study would be necessary to determine the clinical significance of BToV for calf diarrhea.

Bovine coronavirus is historically believed to be a major bovine enteric pathogen causing calf diarrhea, corroborated by pathologic studies (7). However, such a role has
been challenged as some epidemiological studies could not demonstrate a statistically significant association between BCoV infection and calf diarrhea (3, 4, 57). A recent cohort study on Dutch cattle farms even suggested potential opportunistic nature of BCoV infection with previous history of diarrhea (3). In our study, BCoV was found to be significantly associated with calf diarrhea although its association strength with calf diarrhea was relatively weak (OR=2.7). As reported by other investigators, the virus was also detected in some of the fecal samples (12.2%) from healthy calves. While this was initially suspected to be due to fecal shedding of a vaccine virus (54, 55), BRV-A was not detected concurrently in those BCoV-positive samples, noting that commercial vaccines for calf scouring contain both BCoV and BRV-A in a live form. Co-infection or other factors may contribute to diarrhea in association with BCoV infection as levels of BCoV in feces from diarrheic calves were significantly higher than those in feces from healthy calves. Such a quantitative difference may be a useful criterion in determining the clinical significance of BCoV detection during diagnostic investigation.

Bovine enterovirus is commonly present in gastrointestinal tract in cattle and highly prevalent in high-density cattle farms (25, 32). The virus is also known to be stable in the environment (25). Most of BEV infections are subclinical, although gastroenteritis and reproductive disease associated with BEV infection have been reported (5, 6). In our study, detection of BEV did not demonstrate a statistically significant association with calf diarrhea (OR=0.113). In fact BEV was more commonly detected in feces from healthy calves, which supports asymptomatic infection of BEV in bovine gastrointestinal track (25, 32).
Accurate and rapid diagnosis of pathogens of bovine enteric disease is important for quick and appropriate interventions in the field to mitigate losses (36). The detection frequency of BRV-A, BCoV and *C. parvum* was increased by 1.5 to 4.5 times after implementing the BEP, PCR-based testing in ISU-VDL. Such an increase in incidence/prevalence is more likely attributed to higher sensitivity and specificity of BEP than conventional tests and accurately reflects actual epidemiology of these pathogens in the field. Interestingly, the detection frequency of *C. parvum* in diarrhea cases increased by 4.5 times (i.e., from 8.6% to 38.3%) after implementation of BEP, raising awareness of the epidemiological and clinical significance of *C. parvum* in the field. This observation is an example of the bias of test sensitivity on interpretation of infection prevalence or disease prevalence, which, in turn, can misguide veterinary practitioners or producers on disease intervention or animal management on farm. Continuous and frequent evaluation of the performance of diagnostic tests in context of impact on the animal (infection vs. disease) is highly desired to minimize misclassification of data (10).

In conclusion, co-infection of multiple pathogens is common in calf diarrhea cases although clinical significance/role of each pathogen in diarrhea may vary and remains to be further studied for some pathogens. *Cryptosporidium parvum* and BRV-A appear to be the primary enteric pathogens significantly contributing to calf diarrhea under conditions presented in the study. Frequent detection of bovine caliciviruses, such as BNoV and Nebovirus, in feces from diarrheic calves raises the need to pay attention to these viruses with respect to the management of enteric disease on farm. Use of a PCR-based testing panel (e.g., multiplex Real-time PCRs) covering a wide range of known and potential pathogens with defined sensitivity and specificity is strongly recommended for
monitoring/surveillance of populations for diseases, particularly when dealing with multifactorial diseases such as calf diarrhea or bovine respiratory disease complex. Such a screening test for multiple pathogens would be useful for not only studying the host-agent ecology, disease expression and dynamics in a population but also developing an effective intervention strategy for disease control or prevention. In addition, further characterization of pathogens with high rate of mutation on the on-going basis may be necessary to keep a vaccine-based intervention strategy effective.

ACKNOWLEDGEMENT

The authors would like to thank Jessica Boor and Jacqueline Thomas for their excellent assistance in sample collection from VDL submissions. The authors are grateful to Drs. Annette O’Connor and Grant Dewell for their critical review of the manuscript. The study was supported in part by funding from Calf Scouring Fund, VDL R&D fund and USDA (Award No. 2007-35102-18115)

REFERENCES


53. **Sting, R.** 2009. Detection of beta2 and major toxin genes by PCR in Clostridium perfringens field isolates of domestic animals suffering from enteritis or enterotoxaemia. Berl Munch Tierarztl Wochenschr **122**:341-347.


### Table 1. Oligonucleotide sequence of primers and probe used in PCR to detect each target enteric pathogen

<table>
<thead>
<tr>
<th>PCR format</th>
<th>Target pathogen (Primer/ probe sequence[5’-3’])</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Real-time PCR</strong></td>
<td><strong>BCoV</strong> &lt;br&gt;(set 1) &lt;br&gt; f-wd: CTAGTAACCAGGCTGATGTCaatACC &lt;br&gt; rev: GCGGAACCTAGTCGGAATA &lt;br&gt; probe: (FAM/MGB) CGGCTGACATTTCGATC &lt;br&gt; <strong>BRV</strong> &lt;br&gt;f-wd1: TCAACATGGATGTCTGTACCTCCT &lt;br&gt;f-wd2: TCAACATGGATGTCTGTATTCCT &lt;br&gt;f-wd3: TCAACATGGATGTCTTTATTCCT &lt;br&gt; rev1: TCCTCCAGTTGGAAACTCATT &lt;br&gt; rev2: TCCTCCAGTTGGAAATTCATT &lt;br&gt; probe1: (VIC/MGB) TCAAAAATCTTTAAAGATGCTAG &lt;br&gt; probe2: (VIC/MGB) TCAAAAATCTTTAAAGATGCAAG</td>
<td>(9)</td>
</tr>
<tr>
<td><strong>Real-time PCR</strong></td>
<td><strong>BEV</strong> &lt;br&gt;(set 2) &lt;br&gt; f-wd: GCCGTAATGCTGCTATCC &lt;br&gt; rev: GTAGTCTGTTCCGCCYCYRACT &lt;br&gt; probe: (FAM/BHQ1) CGCACAATCCAGTGTACCTACGTAAC</td>
<td>(25)</td>
</tr>
<tr>
<td><strong>Real-time PCR</strong></td>
<td><strong>BVDV</strong> &lt;br&gt;f-wd: GGG NAG TCG TCA RTG GTT CG &lt;br&gt; rev: GTG CCA TGT ACA GCA GAG WTT TT &lt;br&gt; probe: (Cy5/BHQ2) CTTGGGTACCTCTATCTACCA</td>
<td>(33)</td>
</tr>
<tr>
<td><strong>Real-time PCR</strong></td>
<td><strong>BNoV</strong> &lt;br&gt;(set 3) &lt;br&gt; f-wd: CGCCTCCATGTYYGCBTGG &lt;br&gt; rev: TCAGTCATCTTTCATTACAAAATC &lt;br&gt; probe: (Fam/Zen/IABkFQ) TGTGGGAAAGGTTAGTGCACRYC</td>
<td>(63)</td>
</tr>
<tr>
<td><strong>SYBR green</strong></td>
<td><strong>BToV</strong> &lt;br&gt;(set 4) &lt;br&gt; f-wd: TTACTGGYTATTGGGGCMYT &lt;br&gt; rev: AAAGGRGTGCAGTGWGACTTT</td>
<td>(23)</td>
</tr>
<tr>
<td></td>
<td>Sequence Description</td>
<td>Length</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------------------------------------------------------------------------------</td>
<td>--------</td>
</tr>
</tbody>
</table>
| **Real-time PCR**<br>(set 5) | *E. coli K99*<sup>+</sup>-fwd: GCTATTAGTGTCATGGCACTGTAG  
*E. coli K99*<sup>-</sup>-rev: TTTGTTTTGCTAGGCACTGTAG  
*E. coli K99*-Probe: (FAM/BHQ1) ATTTAAACTAAAACCAGCGCCCGGCA | (60)   |
|                | *C. parvum*-fwd: CAAATTGATACCGTTTGTCCTTCTG  
*C. parvum*-rev: GGCATGTCGATTCTAATTCAGCT  
*C. parvum*-probe: (Cy5/BHQ2) TGGCATACATTGTTGTCCTGACAAATTGAA | (19)   |
| **Real-time PCR**<br>(set 6) | *S. typhimurium*-fwd: GCCATGCTGTTCGATG  
*S. typhimurium*-rev: GTTACCGATAGCGGGAAAGG  
*S. typhimurium*-probe: (FAM/BHQ1) TTTTGCACCACMGCCAGCCC | (40)   |
|                | *C. perfringens β*-fwd: TGGAGCGTGAAAGAAACTGTTATTA  
*C. perfringens β*-rev: GGTATCAAAGAGCTAGGGAATAGA  
*C. perfringens β*-probe: (Cy5/BHQ2) CTGAAATTGGAATGGTGCTAACTGGGATAGGACAA | (2)    |
| **Internal control**<br> | P1570: TGGCCCGCAGTATTTCTGATT  
P1642: CAGCTGGCAGCAGGCTTGAG  
P1591M: (Cy3/BHQ1) CCTCGAATCAAACGCCGTTGGAATG | (9)    |
| **Nested RT-PCR**<br> | Nebo-fwd: TTTCATACTGATGGGAYGAYG  
Nebo-rev: GTCACTCATGTTCTTCTCTCTAAT  
nNebo-fwd: CGCTCGTGAGGATACAGA  
nNebo-rev: GCACGGGCTTTCTCTAGAGA | (28)   |
Table 2. Detection frequency of various bovine enteric pathogens among feces from diarrheic and healthy calves in the Midwest and association between positivity and calf diarrhea

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Overall % positive</th>
<th>% positives among diarrheic calves</th>
<th>% positives among healthy calves</th>
<th>p-value</th>
<th>Odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine norovirus</td>
<td>29.1</td>
<td>44.7 (89/199) ^a</td>
<td>16.3 (40/245) ^a</td>
<td>0.042</td>
<td>2.0 (1.002-3.9) ^b</td>
</tr>
<tr>
<td>Cryptosporidium parvum ^c</td>
<td>15.1</td>
<td>33.7 (67/199)</td>
<td>0.0 (0/245)</td>
<td>0.0007</td>
<td>173.0 (8.9-3365.1)</td>
</tr>
<tr>
<td>Bovine coronavirus</td>
<td>20.9</td>
<td>31.7 (63/199)</td>
<td>12.2 (30/245)</td>
<td>0.0034</td>
<td>2.7 (1.4-5.1)</td>
</tr>
<tr>
<td>Bovine rotavirus group A</td>
<td>12.2</td>
<td>27.1 (54/199)</td>
<td>0.0 (0/245)</td>
<td>0.0025</td>
<td>79.9 (4.7-1369.5)</td>
</tr>
<tr>
<td>Nebovirus</td>
<td>0.9</td>
<td>21.6 (43/199)</td>
<td>1.6 (4/245)</td>
<td>0.0001</td>
<td>16.7 (4.0-68.8)</td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>4.1</td>
<td>9.0 (18/199)</td>
<td>0.0 (0/245)</td>
<td>0.0056</td>
<td>80.6 (3.6-1803.7)</td>
</tr>
<tr>
<td>Bovine enterovirus</td>
<td>20.3</td>
<td>5.0 (10/199)</td>
<td>32.7 (80/245)</td>
<td>&lt; 0.0001</td>
<td>0.113 (0.04-0.3)</td>
</tr>
<tr>
<td>Escherichia coli K99 ^f</td>
<td>1.8</td>
<td>4.0 (8/199)</td>
<td>0.0 (0/245)</td>
<td>0.0143</td>
<td>98.4 (2.5-3859.9)</td>
</tr>
<tr>
<td>Bovine torovirus</td>
<td>1.1</td>
<td>2.5 (5/199)</td>
<td>0.0 (0/245)</td>
<td>0.2404</td>
<td>10.4 (0.2-520.3)</td>
</tr>
<tr>
<td>Bovine viral diarrhea virus</td>
<td>0.5</td>
<td>0.5 (1/199)</td>
<td>0.4 (1/245)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Clostridium perfringens toxin β</td>
<td>0.0</td>
<td>0.0 (0/199)</td>
<td>0.0 (0/245)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

^aNumbers in the parenthesis show number of positive feces / number of samples tested

^bNumbers in the parenthesis is 95% confidence interval of the estimated odds ratio.

^cThe bold letters indicate microorganisms detected only in feces from diarrheic calves.
**Table 3.** Association of enteric pathogens with the severity of diarrhea (i.e., watery diarrhea) based on physical appearance of feces

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Number of samples positive for each target</th>
<th>Physical appearance of feces&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p-value</th>
<th>Odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liquid (n=30)</td>
<td>Semisolid (n=69)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine rotavirus group A</td>
<td>26/99&lt;sup&gt;b&lt;/sup&gt; (26.3%)</td>
<td>13/30 (43.3%)</td>
<td>0.013</td>
<td>3.3 (1.3-8.4)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bovine coronavirus</td>
<td>30/99 (30.3%)</td>
<td>10/30 (33.3%)</td>
<td>-&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Bovine viral diarrhea virus</td>
<td>1/99 (1.0%)</td>
<td>1/30 (3.3%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bovine norovirus</td>
<td>42/99 (42.4%)</td>
<td>15/30 (50.0%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bovine torovirus</td>
<td>2/99 (2.0%)</td>
<td>1/30 (3.3%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nebovirus</td>
<td>23/99 (23.2%)</td>
<td>10/30 (33.3%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Salmonella</em> spp.</td>
<td>13/99 (13.1%)</td>
<td>4/30 (13.3%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Escherichia coli</em> K99&lt;sup&gt;+&lt;/sup&gt;</td>
<td>7/99 (7.1%)</td>
<td>4/30 (13.3%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Cryptosporidium parvum</em></td>
<td>28/99 (28.3%)</td>
<td>10/30 (33.3%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Clostridium perfringens</em> toxin β</td>
<td>0/99 (0.0%)</td>
<td>0/30 (0.0%)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Physical appearance of feces was upon receiving of samples with clinical history of diarrhea

<sup>b</sup>Number of positives/number of samples tested

<sup>c</sup>Numbers in the parenthesis is 95% confidence interval of the estimated odds ratio

<sup>d</sup>No significant association was observed
Table 4. Concurrent detection of enteric pathogens in feces from diarrheic calves and their association strength

<table>
<thead>
<tr>
<th>Reference pathogens</th>
<th>Associated pathogens</th>
<th>p-value</th>
<th>Odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine norovirus (BNoV)</td>
<td>BRV-A</td>
<td>&lt; 0.0001</td>
<td>3.6 (1.9-6.8)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C. parvum</td>
<td>&lt; 0.0001</td>
<td>4.2 (2.4-7.4)</td>
<td></td>
</tr>
<tr>
<td>Bovine coronavirus (BCoV)</td>
<td>BRV-A</td>
<td>&lt; 0.0001</td>
<td>3.7 (2.0-6.8)</td>
</tr>
<tr>
<td>Nebovirus</td>
<td>0.0232</td>
<td>2.2 (1.1-4.3)</td>
<td></td>
</tr>
<tr>
<td>Bovine rotavirus group A (BRV-A)</td>
<td>BNoV</td>
<td>0.0005</td>
<td>3.2 (1.7-6.0)</td>
</tr>
<tr>
<td>BCoV</td>
<td>&lt; 0.0001</td>
<td>3.6 (1.9-6.9)</td>
<td></td>
</tr>
<tr>
<td>Salmonella</td>
<td>0.0012</td>
<td>5.9 (2.0-17.1)</td>
<td></td>
</tr>
<tr>
<td>C. parvum</td>
<td>0.0008</td>
<td>3.3 (1.6-6.7)</td>
<td></td>
</tr>
<tr>
<td>Nebovirus</td>
<td>BCoV</td>
<td>0.0496</td>
<td>2.1 (1.0-4.2)</td>
</tr>
<tr>
<td>BToV</td>
<td>0.0066</td>
<td>15.7 (2.2-114.5)</td>
<td></td>
</tr>
<tr>
<td>C. parvum</td>
<td>&lt; 0.0001</td>
<td>9.6 (4.9-18.9)</td>
<td></td>
</tr>
<tr>
<td>Bovine torovirus (BToV)</td>
<td>Nebovirus</td>
<td>0.005</td>
<td>13.5 (2.2-82.8)</td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>BRV-A</td>
<td>0.0013</td>
<td>5.1 (1.9-13.9)</td>
</tr>
<tr>
<td>Cryptosporidium parvum (C. parvum)</td>
<td>BNoV</td>
<td>&lt; 0.001</td>
<td>3.6 (2.0-6.5)</td>
</tr>
<tr>
<td>BRV-A</td>
<td>0.0057</td>
<td>2.7 (1.3-5.6)</td>
<td></td>
</tr>
<tr>
<td>Nebovirus</td>
<td>&lt; 0.001</td>
<td>7.1 (3.5-14.2)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Numbers in the parenthesis is 95% confidence interval of the estimated odds ratio.
Table 5. Comparison of the detection frequency of bovine rotavirus group A (BRV-A), bovine coronavirus (BCoV) and Cryptosporidium parvum (C.parvum) in feces from diarrheic calves before/after use of a PCR-based bovine enteric panel (BEP) in Iowa State University Veterinary Diagnostic Laboratory

<table>
<thead>
<tr>
<th>Year</th>
<th>BRV-A</th>
<th>BCoV</th>
<th>C. parvum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ELISA</td>
<td>ELISA</td>
<td>Direct microscopy (acid fast stain)</td>
</tr>
<tr>
<td>Before BEP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>29.8% (131/440)(^a)</td>
<td>12.4% (50/405)</td>
<td>12.2% (15/123)</td>
</tr>
<tr>
<td>2004</td>
<td>25.8% (102/396)</td>
<td>11.8% (46/391)</td>
<td>12.7% (13/102)</td>
</tr>
<tr>
<td>2005</td>
<td>25.6% (103/402)</td>
<td>9.8% (41/418)</td>
<td>8.4% (12/143)</td>
</tr>
<tr>
<td>2006</td>
<td>18.6% (67/361)</td>
<td>17.0% (24/141)</td>
<td>5.7% (7/123)</td>
</tr>
<tr>
<td>2007</td>
<td>22.7% (123/542)</td>
<td>-</td>
<td>4.5% (5/111)</td>
</tr>
<tr>
<td>average</td>
<td>24.6% (123/2142)</td>
<td>11.9% (161/1355)</td>
<td>8.6% (52/602)</td>
</tr>
</tbody>
</table>

| After BEP |      |      |           |
| 2008  | 40.7% (198/487) | 36.8% (179/487) | 42.5% (207/487) |
| 2009  | 39.5% (213/539) | 27.8% (150/539) | 42.3% (228/539) |
| 2010  | 40.1% (242/603) | 28.4% (171/603) | 38.1% (230/603) |
| 2011  | 29.2% (176/602) | 25.1% (151/602) | 31.6% (190/602) |
| average | 37.2% (829/2231) | 29.2% (651/2231) | 38.3% (855/2231) |

\(^a\)% positive (number of positives /number of total cases)
Figure 1. Quantitative comparison of bovine norovirus (BNoV) and bovine coronavirus (BCoV) shedding in fecal samples from diarrheic (D) and healthy (H) calves. Mean (solid line) and median (dotted line) are shown on a boxplot with 50 percentile distribution. The lower and upper whiskers represent 10th and 90th percentile plot, respectively, and dots represent outliers. Virus shedding level between the 2 groups was compared based on Ct values by the non-parametric Wilcoxon rank-sum test.
Figure 2. Age distribution of diarrheic calves whose feces were positive for one or more enteric pathogens. Animals are classified into 3 age groups: 0-4 weeks, 5-14 weeks and 15-34 weeks of age (upper panel) based on the information provided by submitting veterinarians. Animals at 0-4 weeks of age are further broken down on the weekly basis after birth (lower panel). BRV (bovine
rotavirus), BCoV (bovine coronavirus), BVDV (bovine viral diarrhea virus), BEV (bovine enterovirus), BNoV (bovine norovirus), C. parvum (cryptosporidium parvum) and CptB (clostridium perfringens β toxin).
Figure 3. Frequency (%) of concurrent infection in diarrheic and healthy calves. Numbers (0-6) represent the number of pathogens concurrently detected within each fecal sample. Bovine enterovirus is not included in assessment.
Figure 4. Frequency of viral, bacterial and/or protozoan infections in diarrheic and healthy calves. Viral pathogens included for testing are group A bovine rotavirus, bovine coronavirus, bovine torovirus, bovine norovirus, Nebovirus and bovine viral diarrhea virus. Bacterial pathogens included for testing are *Escherichia coli* K99\(^+\), *Salmonella* spp and *Clostridium perfringens* with β toxin. *Cryptosporidium parvum* (*C. parvum*) is the only pathogen representing the protozoa group.
CHAPTER 6. GENERAL CONCLUSIONS

Although calf diarrhea is a multifactorial disease, this dissertation research focused on better understanding clinical significance of and interaction among historically well-known and emerging bovine enteric pathogens in calf diarrhea. The assessment was conducted in a case-control manner using nucleic acid based assays for all target pathogens to minimize testing bias. From a series of 4 studies, the following conclusions were drawn:

1. Multiple pathogens were involved in calf diarrhea and frequently infected animals concurrently.
2. Historically known major bovine enteric pathogens, such as BRV-A, BCoV, Salmonella, E. coli K99+ and C. parvum, were still commonly and significantly associated with calf diarrhea in this study, suggesting that lack of appropriate maternally derived immunity is still of concern for control of calf diarrhea problem. Both vaccine and treatment options should be considered for effective disease control for these pathogens.
3. Bovine caliciviruses, such as BNoV and Nebovirus, were identified as significant bovine enteric pathogens. BNoV was found to be widely distributed among diarrheic bovine in the Midwest USA with considerable genetic diversity. These observations raise the need to pay attention to these emerging enteric pathogens in conjunction with control of bovine enteric diseases.
4. Although literature suggested zoonotic potential of BNoV, our study could not find an strong evidence supporting interspecies transmission of noroviruses between human beings and bovine.

5. *Clostridium perfringens* type C was not detected in any samples tested, questioning its clinical significance in calf diarrhea. Clinical significance of BEV and BVDV in calf diarrhea was also lacking.

6. Transmission of enteric pathogens through pasture streams is unlikely although bovine feces can be a source for microbial contamination of stream water, which could be minimized by better grazing management.

7. PCR-based panel testing for simultaneous detection of multiple pathogens was a better approach to deal with multi-factorial nature of calf diarrhea and to assess their epidemiology and clinical significance in the disease. In addition, PCR-based panel testing can be applied to periodic monitoring of enteric etiology at the herd level. For some pathogens (e.g., BNoV, BCoV), quantitative nature of testing may be of help to determine its clinical significance in calf diarrhea.

8. Animal-side or at-clinic testing would be ideal when rapid turnaround of test result is desired for immediate intervention of calf diarrhea. However, our study clearly demonstrated that a test kit with improper diagnostic performance can lead to erroneous results misclassifying the index animals. Thorough evaluation of test performance using standard tests must be preceded before a new test gets employed for use by practitioners or diagnostic laboratories.
A few questions still remain to be further studied, although the thesis had produced valuable epidemiological and diagnostic information regarding calf diarrhea.

1. Bovine rotavirus VP4 (P type) and VP7 (G type) genotype should be surveyed because they serve as important protective antigens. A survey for non-group A BRV in conjunction with calf diarrhea may be necessary to assess their role in the disease. In addition, the efficacy of current BRV vaccines should be re-evaluated with contemporary field strains.

2. Bovine caliciviruses (i.e., bovine norovirus and Nebovirus) should be strongly considered as potential enteric pathogens contributing to calf diarrhea disease. Accordingly their clinical significance should be evaluated using animal challenge and/or longitudinal cohort study. Obviously the development of in- vitro cell culture methods for these viruses is required for challenge study, in-depth molecular characterization and vaccine development.

3. Clinical significance of *Clostridium perfringens* type C and other toxins needs to be re-evaluated in conjunction with their quantitatively different fecal shedding pattern in order to better understand its pathogenesis.

4. The high detection frequency of *Cryptosporidium parvum* has raised the need for the development of a better cow-calf management system, effective medical treatment and possibly vaccines.

5. For definitive assessment of roles and interaction of recognized enteric pathogens in calf diarrhea, experimental challenge and/or longitudinal cohort studies should be conducted.
APPENDIX. LONGITUDINAL STUDY OF BOVINE ENTERIC VIRUS INCIDENCE IN MIDWESTERN PASTURE STREAMS FOR 2007 TO 2009 GRAZING SEASONS

A paper to be submitted to Applied and Environmental Microbiology


ABSTRACT

Enterovirus is a biological index for the fecal contamination in water resources, tracing the origin of fecal contaminant because of its host specificity. Using bovine enterovirus (BEV) as a target organism, the occurrence of bovine fecal contamination in pasture streams in Southern Iowa was studied during 2007-2009 grazing seasons. A total of 13 pastures with different stocking systems were employed for the study. Water samples (n=1,274) were collected biweekly from a stream at up-stream, and down-stream sites in each pasture and tested by a multiplex real-time reverse transcriptase-polymerase chain reaction for BEV, bovine coronavirus (BCoV) and group A bovine rotavirus (BRV). Also, the total coliform bacteria count was assessed as the standard of water quality. BEV, BCoV and BRV were detected in 3.91%, 1.12% and 0.48% of the samples, respectively. There was a trend of BEV incidence difference between up- and down-stream sites, implying a dilution effect and/or loading of contaminant from the pasture. Total coliform
bacteria counts did not correlate with BEV incidence as well as cattle presence or stocking density, indicating that other sources of fecal contamination may contribute to bacterial loading of pasture streams. Although the study results suggest that multiple factors affect the quality of pasture streams rather than solely cattle-originated contaminant, appropriate cattle grazing or pasture management practices should be considered to minimize bovine fecal contamination of pasture stream.

**INTRODUCTION**

Cattle grazing practices that allow cattle to congregate near pasture streams result in accumulation of manure as well as loss of vegetative cover and soil compaction near the streams (32, 35). These conditions may cause sediment, phosphorus, and/or pathogen loading of streams by direct deposition of feces or in precipitation runoff (1). Grazing of cattle in streamside areas has been reported to be associated with increased concentrations of coliform bacteria in pasture streams (14, 27).

Coliform bacteria have been considered as the ‘gold standard’ for the estimation of water quality globally (3, 34) as the presence of coliform bacteria is indicative of fecal contamination in water. The numbers of coliform bacteria is commonly used as a criterion to determine the suitability of water for drinking, fishing, recreation, and industrial use (22, 28, 36). However, it is difficult to trace the origin of fecal contamination using coliform bacteria because those bacteria are shed from humans and domesticated or wild animals and their survival period in the environment is shorter than viral indicators such as
enterovirus and adenovirus (19, 26, 31). Therefore, monitoring for the source of
contaminants based on fecal coliform bacteria may not be effective in improving water
quality via elimination of contamination source (11, 19).

Enterovirus, a member of the family *Picornaviridae*, is a non-enveloped
icosahedral virus with a single-stranded positive-sense RNA genome and has been found
in human and many animal species (23, 38). The virus tends to maintain host specificity
for infection (11, 21, 23, 25, 30) and is known to be very stable in both the gastrointestinal
tract and in a wide range of environmental conditions (20, 29). Thus, a large number of
viruses can be shed through feces and maintained without losing the infectivity in the
environment including in a watershed for a long period of time. Because of its
environmental stability and narrow host range, enterovirus has been suggested as a host-
specific index virus of fecal contamination in the water (11, 13, 21, 30, 37). For example,
detection of a human enterovirus, porcine teschovirus (e.g., formally known as group I
porcine enterovirus) or bovine enterovirus in the water can be indicative of fecal
contaminant from humans, pigs and cattle, respectively.

Bovine enterovirus (BEV) is a member of the genus *Enterovirus* in the family
*Picornaviridae* (38). It has been recently proposed to classify BEV isolates into two
molecular clusters (i.e., BEV-A and BEV-B), each of which has two and three
genotypes/serotypes respectively (38). Nonetheless, the viruses are still commonly
classified into two serotypes (BEV-1 and BEV-2). BEV is endemic in cattle populations
and is commonly considered as a non-pathogenic virus, although there have been reports
of gastroenteritis and reproductive disease by BEV infection (5, 6, 23). In contrast, bovine
coronavirus (BCoV) and group A bovine rotavirus (BRV) are known to be major viral enteric pathogens causing calf scouring and shedding in feces from affected animals (9). BCoV has also been implicated in winter dysentery in adult cattle and respiratory disease in all ages of cattle (7, 8, 16, 24). BCoV is an enveloped virus with single-stranded RNA genome whereas BRV is a non-enveloped virus with double-stranded segmented RNA genome. Both viruses have shown their host specificity for cattle.

As stated above, grazing cattle are frequently perceived as a major contributor to fecal contamination of streams, raising a public health concern. However, the origin of contaminants in those water sources has not been critically studied. The following study was conducted for 3 years, from 2007 to 2009, to survey the prevalence of bovine enteric viruses (BEV, BCoV and BRV) in addition to coliform bacteria in pasture streams during grazing season to evaluate the effects of cattle grazing on the source of microbial contamination of pasture streams and develop grazing management strategies which can reduce water contamination by livestock.

MATERIALS AND METHODS

Overview of study. Thirteen pastures were employed as study farms in Southern Iowa and water samples (n=1,274) were collected from up-stream and down-stream sites on pasture streams for 3 years during 2007 - 2009. Biological factors (i.e., BEV, BCoV, BRV, coliform bacteria, and E. coli O157:H7) related with water quality or waterborne disease were tested with multiplex real-time reverse transcription-polymerase chain reaction (rRT-
PCR) and bacterial culture assay. In addition, other non-biological factors, such as cattle movement or distribution, cattle stocking density, cattle presence or absence, and rainfall, were observed with cattle stocking records and HOBO weather stations (Onset Computer Corporation, Bourne, MA), respectively, in this study. The correlation within biological factors (e.g., coliform bacteria versus BEV) as well as between factors (e.g., biological factors versus other factors) were analyzed.

Pasture characteristics, cattle stocking densities and microclimate recording.

Streams passing through 13 pastures on 12 cooperating farms in the Rathbun Lake watershed in southern Iowa were identified as appropriate for the project. One farm had two connected pastures. Pastures ranged in size from 2.8 to 107.2 hectares (ha) with stream reaches of 306 to 1,778 meters (m) that drained watersheds of 252 to 5,660 ha. Pastures were continuously or rotationally stocked with beef cows and the cows had unlimited access to pasture streams, exceptionally one pasture stream was restricted cattle grazing as Conservation Reserve Program. Most of producers had spring-calving herds with a few producers having both spring- and fall-calving herds. Grazing management of the cows was controlled by the manager of each farm and the system was not altered by the researchers. Managers of these operations recorded the number of cows, heifers, and bulls stocked in these pastures as they entered and were removed from the pasture from November, 2006 to November, 2009. These record books were used to determine if cattle presence/absence in pastures was associated with detection of 3 bovine enteric viruses
(BEV, BCoV and BRV) and bacteria (coliarm bacteria and *E. coli* O157:H7) in the samples.

In addition, yearly stocking densities were calculated from record books and used to regress against annual total coliforms count and viral incidence (i.e., BEV, BCoV and BRV) per year of upstream and downstream water sampled. Because of the variable pasture sizes and stream reaches, and numbers of cows, heifers, and bull stocked in pastures across years, comparison across farms were made by adjustments of the procedure of by Allen et al., 1991 (2) to calculate animal units as follows:

\[
\text{Annual stocking rate} / (\text{pasture ha}) \text{ or (stream m)} = [(1.00\text{*cow-days}) + (0.84\text{*heifer-days}) + (1.30\text{*bull-days})] / (\text{pasture ha}) \text{ or (stream m)}
\]

where, $ha$ = hectare and $m$=meter.

Six HOBO weather stations equipped with rain gauges recorded rainfall from March to November of each study year. The six weather stations were strategically distributed and placed on a farm located within at least two miles of other farms throughout the watershed. Rainfall amounts were recorded at 10-min interval for the duration of the experiment. Data were used to assess the rainfall effect on the incidence of the bovine enteric viruses and/or coliform bacteria from the stream.

**Seasonal analysis.** Samples were classified as those collected during spring, summer and fall. For each year, spring, summer, and fall samples were collected from March to May, June to August, and September to November, respectively.
Sample collection. From May 2007 to November 2009, water samples were collected biweekly from all selected pasture streams. Water was sampled at the upstream and downstream locations for each stream from the middle of the flowing stream, at approximately half of the distance from the water surface to the streambed in order to collect water samples from the mean velocity flow of the stream. Water collection was made using an adjustable (2.4-7.3 m) swing sampler (Ben Meadows®, Janesville, WI) with an attached 500-ml HDPE bottle. Prior to sample collection, the HDPE bottle was rinsed twice downstream of the intended sampling location to avoid of turbid water where sample collected and, thereafter, thoroughly dried before sampling additional locations to prevent possible water contamination from site to site. Samples collected from the stream were transferred, while still in the field, to 532 ml (18 oz.) WHIRL-PAK® bags (Nasco, Fort Atkinson, WI).

Water samples, approximately 500ml of water, were collected at each end of the stream (i.e., upstream and downstream) and analyzed for the presence of the bovine enteric viruses and bacteria. Only samples from flowing streams or adequate water depth were collected. Consequently, no sample was collected if there was no flow or the stream was dry because of regional weather conditions. A total of 25 samples were collected during each sampling event. Because one farm had two adjacent pastures, three water samples were collected at upstream, midstream (i.e., the stream location where the two pastures connected) and downstream locations. The midstream sample served as the downstream location for the preceding pasture and upstream location for the following pasture. All
samples were kept in an iced cooler box once collected for transport to the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL), Ames, Iowa for sample submission on same day and remained at 4°C until next day processing.

**Sample processing.** For bacterial testing, water samples were used as received without any further processing. For viral PCR testing, each of the submitted water samples (n = 1,254) was transferred into a 50-ml polyethylene tube (Falcon®, Franklin lake, NJ) for centrifugation at 3,000 × g for 20 min. Then 12 ml of each supernatant were pipetted to an ultracentrifuge tube (Ultra-Clear™, Beckman, Brea, CA) for ultracentrifugation at 126,444 × g for 120 min. One ml of 0.01 M phosphate-buffered saline (PBS, pH 7.2) was added to the resulting pellet after the supernatant was discarded, and then the tube was vigorously vortexed. The processed water samples were kept at -80°C until tested.

**Multiplex real-time reverse transcription-polymerase chain reaction (rRT-PCR) for bovine enteric viruses.** Viral RNA was first extracted from each of the processed water samples using a MagMAX™ viral isolation kit (Ambion, Austin, TX) as per the protocol recommended by manufacturer. In brief, 50 µl of each sample, 130 µl of lysis/binding solution, and 20 µl of paramagnetic beads were mixed together in a 96 deep-well microplate (VWR International, Radnor, PA). The remaining extraction steps were done on KingFisher® 96 semi-automated magnetic particles processor (Thermo Fisher Scientific Inc., Waltham, MA) by following the protocol provided by the manufacturer.
A multiplex rRT-PCR panel, which was previously developed in our laboratory for simultaneous detection of 5 major bovine enteric pathogens associated with calf diarrhea (i.e., BCoV, BRV, *E. coli* K99+, *Cryptosporidium parvum*, *Salmonella* spp.) (9), was applied to the amplification of nucleic acid of BEV, BCoV, and BRV using AgPath-ID™ Multiplex RT-PCR Kit (Ambion, Austin, TX). For detection of BEV, published primers and probes (20) were adopted and modified after alignment analysis in order to detect all BEV sequences available in Genebank (NCBI database). Modified BEV primers and probes (Table 1) were added to the PCR panel and evaluated on various BEV strains. PCR reaction was followed as per the manufacturer’s recommended protocol in 25 µl reaction volume containing 25 µM each primer and probe, and 8 µl of extracted RNA template on ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Austin, Austin, TX). The thermal cycling condition was as follows: reverse transcription for 10 min at 45°C, denaturation for 10 min at 95°C, and 40 alternated heat cycles of denaturation at 95°C for 15 sec and primer annealing/extension at 60°C for 60 sec. Samples with a threshold cycle (Ct) of 40 cycles or less were considered positive.

**Plasmid standards.** To evaluate and optimize the real-time RT-PCR assays, the standard positive controls were constructed. The VR-754 strain of BEV, Nebraska strain of BCoV and NCDV strain of BRV were amplified with the primers listed in Table 1 and were cloned into a pCR®2.1 Vector of TA Cloning® Kit (Invitrogen, Carlsbad, CA) as per manufacturer’s instructions. Insertion of the correct target into the vector was verified by sequencing. Plasmid DNA was purified using the QIAprep® Spin Miniprep Kit (QIAGEN,
Germantown, MD) and the DNA amount measured by spectroscopic analysis. To
determine analytic sensitivity of the PCR, each plasmid DNA preparation was diluted by
10-fold serial dilution technique and tested by the PCR in triplicates.

**Bovine enterovirus reference strains.** BEV-1 (LCR4 and M2 strains) and BEV-2
(PS89, PS83 and PS87 strains) were purchased from the American Type Culture
Collection (ATCC) (Manassas, VA). BEV-Oklahoma US (OKA) strain, which was
reported to be BEV-1 (5), was kindly provided by Dr. Jeremiah Saliki at University of
Georgia (Athens, GA). All viruses were propagated and titrated in Mardin-Darby Bovine
Kidney cells (ATCC, Manassas, VA). The viruses were used for evaluating the
performance of the multiplex rRT-PCR panel for BEV detection. The multiplex rRT-PCR
was performed in parallel to the single rRT-PCR (i.e., BEV detection) or duplex rRT-PCR
(i.e., both BCoV and BRV detection).

**Total coliform bacteria.** Samples were analyzed for total coliform bacteria at the ISU-
VDL. Briefly, 100 ml of each water sample were transferred into a bottle containing 2 ml
of sodium thiosulfate. Each of the prepared samples was then filtered on a sterile 0.22 µm
filter membrane (Millipore, Billerica, MA) which was subsequently cultured on a plate
containing Bacto™ mEndo Broth MF media (Difco, Lawrence, Kansas) at 35°C for 24
hours. Red colonies with golden-green metallic sheen were considered as members of the
coli-form bacteria group. The number of visible colonies was counted for each sample and expressed as colony forming unit (CFU) per 100ml.

Although a total of 1,254 samples were collected and tested, only 1,066 samples resulted in numerical values which could be analyzed. The 188 samples resulted with ‘too numerous to count’ (TNTC); therefore, those 188 samples were removed from data analysis.

*Escherichia coli O157:H7 detection.* One hundred ml of each stream water sample was filtered through a 0.22 µm filter unit. The filter was then layered onto 2 ml of Bacto™ mEndo broth MF (Difco) and incubated overnight at 37°C. Bacterial growth was transferred onto a MacConkey agar plate. After overnight incubation, individual colonies were frozen in Brain Heart Infusion (BHI) broth containing 30% glycerol. One hundred µl from the BHI/glycerol broth was added to Tryptic Soy Broth and incubated overnight at 37°C. Broth cultures were plated onto sorbitiol MacConkey agar containing cefiximine (50 ng/ml), tellurite (2.5 µg/ml), and vancomycin (8 µg/ml). The plates were incubated overnight. Characteristic tan colonies were tested using an O157:H7 specific latex agglutination kit.

*Data analyses.* Detections of bovine enteric viruses and total coliform bacteria from water samples were analyzed with the multiple factors, such as cattle presence or absence, time (e.g., monthly and seasonally) and rainfall, by using the Chi-Square test of FREQ
The numerical data of total coliform bacteria was transferred to binominal data for evaluation of correlation between BEV and coliform bacteria. According to EPA’s criteria for the measuring recreation water quality, above the 235 CFU/100 ml of coliform bacteria count was input as 1 (i.e., not suitable) and less than 235 CFU/100 ml of coliform bacteria count as 0 (i.e., suitable) for binomial factor.
In all cases, a \( p \) value < 0.05 was considered significant, whereas a tendency was considered with a \( p \) value of \( \geq 0.05 \) to < 0.15 and a \( p \) value > 0.15 was defined as not significant.

**RESULTS**

**Optimization of multiplex real-time RT-PCR.** The constructed plasmids from the BEV, BCoV and BRV control strains with known viral copy numbers were serially diluted 10-fold in cell culture media and used to optimize the multiplex rRT-PCR. The PCR panel was able to simultaneously detect each of the reference strains specifically without cross reactivity or any false-positive results. Also, all of the seven different BEV strains were amplified with the multiplex rRT-PCR. Standard curves generated using the serial dilutions of each virus showed correlation coefficients of 0.9948 -0.9956 with slopes of 3.29 to 3.47 (Fig1), suggesting that the PCR performed excellently for each target pathogen with good fidelity. The limit of detection for each plasmid standard was 6.2, 4.2 and 2.5 genomic copies for BEV, BCoV and BRV, respectively.

**Detection of bovine enteric virus and bacteria in pasture streams.** Among total of 1,254 water samples, BEV, BCoV, BRV, and *E. coli* O157:H7 were detected in 3.91%, 1.12%, 0.49% and 0.0% of the samples, respectively (Table 2). There was a tendency of difference in the BEV incidence rates between up- and downstream sites \( (p = 0.1053) \),
being higher in upstream than downstream sites. Such a difference was not observed with BCoV and BRV.

Mean coliform bacteria counts (±SEM) for up- and downstream samples (n=1,066) were 1,269 (± 102) and 1,417 (± 103) CFU/100 ml respectively (Table 3), but the difference between up- and downstream samples was not statistically significant ($p = 0.3090$). In contrast, differences in total coliform bacteria count were observed between pastures ($p = 0.0179$), but large variations in concentrations occurred between up- and downstream samples.

Seasonal differences in viral and/or bacterial detection were noted (Fig 2 and 3). Incidence of BEV detection was significantly ($p < 0.0001$) higher in summer and fall than spring, as expected in response to increased ambient temperatures resulting in congregation of more grazing cattle in or near the surface waters. In contrast, incidence of BCoV detection was significantly ($p < 0.0001$) higher in fall than spring and summer. BRV incidence did not show any seasonality. Both of these observations were unexpected because most of participating producers had spring-calving herds. The coliform bacteria count was highest in spring and decreased in the summer and fall.

**Correlation between cattle presence or stocking density and virus detection.** The presence or absence of cattle on the pasture area was compared to the incidence of virus detection in both up- and downstream sites to study the effect of cattle grazing on feces and pathogen loading of pasture streams. As expected, rate of BEV incidence in stream water
was higher when cattle presence on pasture was noted on the day of sampling on 0D ($p = 0.0130$) and up to 6 days prior to sampling day ($0.0283 \leq p \leq 0.0710$), implying that grazing cattle may contribute to fecal contamination of pasture streams (Fig. 4). However, incidence of BCoV and BRV did not show a positive correlation with the cattle presence on the pasture area.

Even though BEV incidence had a positive correlation with cattle presence in pasture, no linear, quadratic, or cubic relationship existed between viral incidence and the stocking density measures evaluated (Fig. 5 and 6). Furthermore, total coliform bacterial count also was not correlated to the stocking density measures (Fig. 7 and 8).

**Relationship of rainfall and microbial incidence.** The BEV incidence in water samples was higher at the downstream site when there was no rainfall within 72H prior to sampling (Fig. 9). However, there was no difference in BEV incidence between up- and downstream when there was rainfall within 72H of sampling. These results imply that rainfall had a distribution effect on BEV in the pasture stream. BCoV and BRV in stream water samples had no relation with rainfall probably due to inconsistent shedding of the viruses. Total coliform bacterial counts were increased at both ends of stream when there was rainfall within 72H.

**Correlation between BEV and coliform bacteria.** To evaluate the effect of bovine origin fecal contamination to the water quality, the correlation between BEV and coliform
bacteria was analyzed (Fig 10). No relationship was identified ($p=0.24$) between BEV and total coliform bacteria incidence when 235 CFU/ml or higher was used as cut-off for the impairment of water quality, implying that the measured water quality was not directly affected by bovine feces and that there may be other sources contributing coliform bacteria pasture streams.

**DISCUSSION**

A large portion of lands in the Midwest USA is used for agricultural purposes (33). Recently, livestock production has been cited as one of environmental contaminants due to increased production and density (14). A field-based 3-year longitudinal study was conducted to assess microbiological loads in streams passing through 13 different pastures of 12 cow-calf farms in the Rathbun Lake watershed in southern Iowa. GPS monitoring of cattle distribution and movement on pastures during a companion study demonstrated that cattle spent approximately 20% of their time in areas near or in pasture streams for drinking or thermoregulation depending on pasture size (4). This observation, along with detection of BEV (biomarker for bovine feces) in pasture streams, the positive correlation between BEV incidence and the presence of cattle on pasture, and apparent coincidence between the seasonality of BEV incidence (i.e., higher in summer and fall than spring) and increased congregation of grazing cattle near streams support the hypothesis that grazing cattle can directly and indirectly (e.g., runoff of raining) contribute to fecal contamination of stream water (14, 20, 32).
Grazing cattle have been reported to contribute to increased number of total coliform bacteria in streams passing through pastures, resulting in impairment of water quality (27). However, our study observations indicate otherwise. No direct correlation between BEV incidence and coliform bacteria incidence/level was observed. Furthermore, stocking density of cattle was not correlated with incidences and/or levels of BEV and coliform bacteria in pasture streams. These observations suggest that other factors contribute to the concentrations of coliform bacteria in water. Wild animals (e.g., deer, goose), human wastewater or aquatic animals can be a source of the fecal coliform bacteria (11, 12, 14, 26, 31). Manure fertilizer applied to crop field can also be a contributing factor. Some of stream areas were close to crop producing fields. The application of manure fertilizer to the field would affect increasing fecal coliform bacteria in the pasture steam. However, no correlation was observed between the time of applying manure fertilizer (e.g., mostly fall season manuring) and the seasonal pattern of coliform bacteria level (e.g., high in spring and summer season), rather more complexity would influence the coliform bacteria level in stream. While further study remains to be done to determine contributing factors, our study confirms the limitation of fecal coliform bacteria for tracing the origin of a fecal contamination source. Use of an appropriate species-specific biomarker will be necessary to identify the correct source of contamination and give a solution for removing or minimizing the source.

Unlike BEV, BCoV and BRV are major bovine enteric viral pathogens causing calf diarrhea and are known to be transmitted through the fecal-to-oral route (9). Thus, it would be possible that these viruses could be spread through pasture stream promoting
waterborne disease. On the contrary, our 3-year longitudinal study on 13 pastures revealed a low incidence rate (≤ 1.12%) of these viral pathogens in pasture streams and a lack of correlation between seasonal pattern of viral incidence and calving season. These results suggest that waterborne transmission of BCoV and BRV is unlikely in the field. A relatively low incidence rate (3.91%) was also observed with BEV detection in our study even though the virus is generally considered to be common in cattle population.

Considering potential dilution effects of pasture streams on the target viral agents, one can argue that the small amount (12ml) of stream water processed for PCR testing may account for the observed low incidence rate of these agents. Some previous studies used a higher amount (e.g., 40 ml) of water to process for testing (17, 25). However, it should be noted that analytic sensitivity of the PCR assay used in the study was estimated to be 4.2 and 2.5 genomic copies for BCoV and BRV respectively. Theoretically the sample should have been positive for one or both agents if stream water had at least one detectable BCoV or BRV particle in 12ml. Therefore it is less likely that the low prevalence was attributed to sample volume. There are two plausible factors which may have led to the low incidence of bovine viral pathogens. First, the density of animals and grazing management in each pasture may have been adequate to lower level of the viruses on pastures or in streams. Second, the fact that the majority of grazing animals on pastures were adult cattle may have led to low incidence of BCoV and BRV since these pathogens cause scouring mainly in newborn calves and adult cattle may shed virus, if any, at a much lower rate and frequency. Nevertheless, it was interesting that BCoV incidence was higher than BRV. It was probably because BCoV infection can also be a cause of respiratory disease and winter
dysentery in adult cattle (7, 8, 16). Overall, it appears that grazing cattle do not impose a serious risk for disease transmission via pasture stream.

In general, high density of cattle grazing has been considered as a main reason for environmental contamination by fecal loading (27). However, this study did not demonstrate a direct relationship between cattle stocking density and the incidence of BEV or the concentrations of total coliform bacteria, suggesting that multiple factors are involved in impairing the quality of the pasture stream water. For example, rainfall can play a role as mechanical transport of fecal contaminant from one site to the other site (10, 27). In our study, total coliform bacteria counts in stream water were higher within 72 hours after rainfall. This bacterial loading was probably the result of precipitation runoff of feces particularly from non-vegetative pasture areas close to the stream, floating of coliform bacteria sediment or transporting coliform bacteria from other sources such as human wastewater. Besides, the location, timing, and length of cattle grazing have been reported to be important factors affecting the water quality in conjunction with grazing pasture (35). The difference in pasture character (e.g., erosion, vegetation), pasture management style, and the cattle grazing practice of each farm also can influence the water quality (32). Even though the detection rate of bovine enteric viruses in pasture streams was much lower than anticipated from previous reports, our study did demonstrate that grazing cattle contributed to fecal loading and microbial contamination of pasture stream. Therefore, implementation of practices which minimize cattle or wild animal access to areas close to pasture streams should be considered to reduce the risks of bovine fecal contamination of pasture streams. Because increased environmental temperature is known
to induce the cattle congregation near because of thirst and increasing body heat (15), supplying drinking water source as well as provide a wide shading area (i.e., tree) away from pasture streams could reduce cattle presence near pasture streams (18). Furthermore, limiting cattle access to stabilized crossings or to a riparian paddock in a rotational stocking system will also reduce cattle presence in pasture streams (15). All those factors affecting cattle movement could be controlled through grazing management.

ACKNOWLEDGEMENTS

The authors would like to thank the cooperating producers for data collection books and assess to collect samples, USDA-NRCS personnel for assistance with collecting water samples, virology and bacteriology technical staff of the Veterinary Diagnostic Laboratory at Iowa State University for assistance in testing the samples, and Garritt Page and Dr. Wang for assistance with statistical analysis. The study was in part supported by funding from the USDA Cooperative State Research, Education, and Extension Service (Award No. 2007-35102-18115) and by the Iowa Calf Scour Fund made from donation by Dr. Rex Whelm.

REFERENCES


winter dysentery coronavirus strains in calves and RT-PCR and nested PCR for their detection. Arch Virol 146:2401-2419.


enterovirus in biological and environmental samples by a highly sensitive real-time reverse transcription-PCR. Appl Environ Microbiol 71:3536-3543.


**TABLE 1.** Oligonucleotide sequences of primers and probes used in multiplex real-time PCR to detect bovine coronavirus (BCoV), group A bovine rotavirus (BRV) and bovine enterovirus (BEV)

<table>
<thead>
<tr>
<th>Primer or probe</th>
<th>Sequence (5’-3’)</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCoV-fwd</td>
<td>CTAGTAACCAGGCTGATGTCAATACC</td>
<td>87</td>
<td>(9)</td>
</tr>
<tr>
<td>BCoV-rev</td>
<td>GGC GGAAACCTAGTCGGAATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCoV-probe (FAM/MGB)</td>
<td>CGGCTGACATTCTCGATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRV-fwd1</td>
<td>TCAACATGGATGTCCTGTACTCCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRV-fwd2</td>
<td>TCAACATGGATGTCCTGTATTCCCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRV-fwd3</td>
<td>TCAACATGGATGTCCTTTATTCCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRV-rev1</td>
<td>TCCTCCAGTTTGGAACTCATT</td>
<td>155</td>
<td>(9)</td>
</tr>
<tr>
<td>BRV-rev2</td>
<td>TCCCCCAGTTTGGAAATTCATT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRV-rev3</td>
<td>CCCTCCAGTTTGGAAATTCATT</td>
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<td></td>
</tr>
<tr>
<td>BRV-probe1 (VIC/MGB)</td>
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<td></td>
</tr>
<tr>
<td>BRV-probe2 (VIC/MGB)</td>
<td>TCAAAAAACTCTTTAAAGATGCAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BEV-fwd</td>
<td>GCCGTGAATGCTGCTAATCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BEV-rev</td>
<td>GTAGTCTGTTCCGCCYCYRACT</td>
<td>93</td>
<td>(20)</td>
</tr>
<tr>
<td>BEV-probe (CY5/BHQ-2)</td>
<td>CGCACAATCCAGTGGCTACGTCGTAAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**TABLE 2.** Incidence of bovine enterovirus (BEV), bovine coronavirus (BCoV), group A bovine rotavirus (BRV) and *E. coli* O157:H7 in pasture stream water samples collected at upstream and downstream locations from 13 pastures on 12 farms during the 2007, 2008 and 2009 grazing seasons

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. of samples</th>
<th>No. of positives</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEV</td>
<td>1,254</td>
<td>49</td>
<td>3.9</td>
</tr>
<tr>
<td>BCoV</td>
<td>1,254</td>
<td>14</td>
<td>1.1</td>
</tr>
<tr>
<td>BRV</td>
<td>1,254</td>
<td>6</td>
<td>0.5</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>1,254</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
TABLE 3. Mean concentration of total coliforms in water samples (n = 1,066) collected at up- and downstream locations from 13 pastures on 12 farms during the 2007, 2008, and 2009 grazing seasons

<table>
<thead>
<tr>
<th>Pasture</th>
<th>Upstream</th>
<th>Up SE</th>
<th>Downstream</th>
<th>Down SE</th>
<th>Average</th>
<th>Ave SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1085.12</td>
<td>361.07</td>
<td>1551.16</td>
<td>361.07</td>
<td>1318.14</td>
<td>255.32</td>
</tr>
<tr>
<td>2</td>
<td>1821.43</td>
<td>365.34</td>
<td>2689.95</td>
<td>365.34</td>
<td>2253.69</td>
<td>258.34</td>
</tr>
<tr>
<td>3</td>
<td>1431.90</td>
<td>365.34</td>
<td>867.32</td>
<td>369.77</td>
<td>1149.61</td>
<td>259.91</td>
</tr>
<tr>
<td>4</td>
<td>862.44</td>
<td>369.77</td>
<td>956.19</td>
<td>365.34</td>
<td>909.31</td>
<td>259.91</td>
</tr>
<tr>
<td>5</td>
<td>862.62</td>
<td>365.34</td>
<td>1251.90</td>
<td>365.34</td>
<td>1057.26</td>
<td>258.34</td>
</tr>
<tr>
<td>6</td>
<td>1117.67</td>
<td>361.07</td>
<td>1311.90</td>
<td>365.34</td>
<td>1214.79</td>
<td>256.83</td>
</tr>
<tr>
<td>7</td>
<td>1802.86</td>
<td>365.34</td>
<td>1620.67</td>
<td>379.14</td>
<td>1711.76</td>
<td>263.26</td>
</tr>
<tr>
<td>8</td>
<td>1151.25</td>
<td>374.37</td>
<td>1445.79</td>
<td>384.09</td>
<td>1298.52</td>
<td>268.18</td>
</tr>
<tr>
<td>9</td>
<td>1136.59</td>
<td>369.77</td>
<td>1227.91</td>
<td>361.07</td>
<td>1182.25</td>
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<tr>
<td>10</td>
<td>1780.25</td>
<td>374.37</td>
<td>1691.50</td>
<td>374.37</td>
<td>1735.88</td>
<td>264.72</td>
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<tr>
<td>11</td>
<td>837.07</td>
<td>369.77</td>
<td>1188.97</td>
<td>379.14</td>
<td>1013.02</td>
<td>264.80</td>
</tr>
<tr>
<td>12</td>
<td>1575.00</td>
<td>374.37</td>
<td>1445.00</td>
<td>374.37</td>
<td>1510.00</td>
<td>264.72</td>
</tr>
<tr>
<td>13</td>
<td>1032.63</td>
<td>384.09</td>
<td>1172.50</td>
<td>374.37</td>
<td>1102.57</td>
<td>268.18</td>
</tr>
</tbody>
</table>

Average 1268.99 102.42 1416.67 102.83

*a,b,z* Within a column, least squares means without a common subscript differ (*p* < 0.05)
FIG. 1. Multiplex detection of group A Bovine rotavirus (BRV), Bovine coronavirus (BCoV) and Bovine enterovirus (BEV) RNAs demonstrating excellent fidelity of PCR ($R^2 = 0.9948 - 0.9956$). All of the 3 plasmids containing target sequence of each virus with known copy number were mixed and serially diluted 10-fold for simultaneous detection by multiplex rRT-PCR. Y axis indicated cycle threshold (Ct) values. Each regression line was constructed based on 3 repeated measurements.
<table>
<thead>
<tr>
<th>Month</th>
<th>Mar</th>
<th>Apr</th>
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<th>Jun</th>
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<th>Aug</th>
<th>Sep</th>
<th>Oct</th>
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<td>(cfu/100ml)</td>
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<td>500</td>
<td>1000</td>
<td>1500</td>
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<td>2500</td>
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<th>Season</th>
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<th>Summer</th>
<th>Fall</th>
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</thead>
<tbody>
<tr>
<td>(cfu/100ml)</td>
<td>2000</td>
<td>1500</td>
<td>500</td>
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**FIG. 2.** Monthly and seasonal changes of coliform bacteria counting in water samples (n = 1,254) collected at upstream and downstream locations from 13 pastures on 12 farms during the 2007, 2008, and 2009 grazing seasons.
FIG. 3. Monthly and seasonal incidences of bovine enterovirus, bovine coronavirus, and bovine rotavirus (group A) in water samples (n = 1,254) collected at upstream and downstream locations from 13 pastures on 12 farms during the 2007, 2008, and 2009 grazing seasons.
FIG. 4. Incidence of bovine enterovirus (BEV), bovine coronavirus (BCoV) and bovine rotavirus (BRV) in upstream and downstream in relation to cattle presence on pastures on the day of sampling (0) and 3- and 6-days (D) prior to sampling during the 2007, 2008 and 2009 grazing seasons.
FIG. 5. Mean yearly incidences of bovine enterovirus (BEV), bovine coronavirus (BCoV), and bovine rotavirus (BRV) in water samples (n=1,254) by annual stocking rate\textsuperscript{1} per pasture area (ha).

BEV: $y = 3.84234 + 0.00653x$ ($r^2 = 0.0029$)

BCoV: $y = 0.63927 + 0.00764x$ ($r^2 = 0.0365$)

BRV: $y = 0.35671 + 0.00181x$ ($r^2 = 0.0053$)

\textsuperscript{1}Annual stocking rate is in form of annual cow-days per pasture area (ha).
FIG. 6. Mean yearly incidences of bovine enterovirus (BEV), bovine coronavirus (BCoV), and bovine rotavirus (BRV) in water samples (n=1,254) by annual stocking rate\(^1\) per stream reach (m).

BEV: \( y = 3.82348 + 0.03935x \) \( (r^2 = 0.0028) \)

BCoV: \( y = 0.44736 + 0.06243x \) \( (r^2 = 0.0652) \)

BRV: \( y = 0.15208 + 0.03013x \) \( (r^2 = 0.0392) \)

\(^1\)Annual stocking rate is in form of annual cow-days per stream reach (m).
FIG. 7. Mean yearly total coliforms in water samples (n=1,066) by annual stocking rate\(^1\) per pasture area (ha).

Upstream: \(y = 1080.60465 + 1.20047x\) \((r^2 = 0.0064)\)

Downstream: \(y = 1166.13390 + 2.03769x\) \((r^2 = 0.0131)\)

\(^1\)Annual stocking rate is in form of annual cow-days per stream reach (m).
FIG. 8. Mean yearly total coliforms in water samples (n=1,066) by annual stocking rate\(^1\) per stream reach (m).

Upstream: \( y = 1154.57743 - 0.23631x \) \((r^2 = 0.0000)\)

Downstream: \( y = 1312.39530 - 2.39830x \) \((r^2 = 0.0005)\)

\(^1\)Annual stocking rate is in form of annual cow-days per stream reach (m).
FIG. 9. Incidence of bovine enterovirus (BEV), bovine coronavirus (BCoV) and bovine rotavirus (BRV) in upstream and downstream in relation to rainfall in pastures on the day of sampling (0), and 24-, 48-, and 72-hours (H) prior to sampling during the 2007, 2008 and 2009 grazing seasons.
FIG. 10. Comparison of seasonal coliform bacterial level to seasonal incidence of bovine enterovirus (BEV) in pasture streams during the 2007, 2008 and 2009 grazing seasons.

<table>
<thead>
<tr>
<th>Season</th>
<th>Spring (cfu/100ml)</th>
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<th>Fall (cfu/100ml)</th>
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<table>
<thead>
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<th>Summer (BEV incidence rate %)</th>
<th>Fall (BEV incidence rate %)</th>
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ACKNOWLEDGEMENT

I would first like to thank my advisor and mentor Dr. Kyoung-Jin Yoon for his guidance and support both academically and personally. I would also like to acknowledge my POS committee members Drs. Annette O’Connor, Grant Dewell, James Roth and Vickie Cooper for everything they have shared to broaden my eyes to not only science but also the life in the world.

And of course I would like to acknowledge my lab mates Dong Sun, Hai Hoang, Fanghong Zhou, Sara Nezami and Shahan Azeem, and visiting scholars Drs Jaeik Han and Shao-Yi Su. Just staying together with them itself was extremely happy and helpful.

I pray for my thanks to the Korean Catholic Student Community, and all brother and sister in St. Thomas Aquinas Catholic Church, Ames, Iowa for their love and prayer.

Lastly but not the least, I would like to thank my wife (also my best friend), Hyewon Lee for her endless love and devotion to our family, and my son Ian Joonhyeop Cho for his pure smile changed everything into beautiful goodness. My parents Chungpo Cho and Hyunsoon Choi, and my parents in law Seongso Lee and Yongeun Lee were my best support at times of need.