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Review

An overview of calf diarrhea - infectious etiology, diagnosis, and intervention

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Calf diarrhea is a commonly reported disease in young animals, and still a major cause of productivity and economic loss to cattle producers worldwide. In the report of the 2007 National Animal Health Monitoring System for U.S. dairy, half of the deaths among unweaned calves was attributed to diarrhea. Multiple pathogens are known or postulated to cause or contribute to calf diarrhea development. Other factors including both the environment and management practices influence disease severity or outcomes. The multifactorial nature of calf diarrhea makes this disease hard to control effectively in modern cow-calf operations. The purpose of this review is to provide a better understanding of a) the ecology and pathogenesis of well-known and potential bovine enteric pathogens implicated in calf diarrhea, b) describe diagnostic tests used to detect various enteric pathogens along with their pros and cons, and c) propose improved intervention strategies for treating calf diarrhea.

Keywords: calf diarrhea, etiology, intervention

Introduction

Calf diarrhea (also known as calf scouring) is a commonly reported disease and a major cause of economic loss to cattle producers. The 2007 National Animal Health Monitoring System (NAHMS) for U.S. dairy [135] reported that 57% of weaning calf mortality was due to diarrhea and most cases occurred in calves less than 1 month old. A similar mortality rate (53.4%) for dairy calves due to calf diarrhea was recently reported in Korea [61]. The economic loss associated with calf death in Norway where calf production is 280,000 heads per year was estimated to be approximately 10 million US dollars in 2006 [103].

Calf diarrhea is attributed to both infectious and non-infectious factors [8,62]. Multiple enteric pathogens (*e.g.*, viruses, bacteria, and protozoa) are involved in the development of this disease. Co-infection is frequently observed in diarrheic calves although a single primary pathogen can be the cause in some cases. The prevalence of each of pathogen and disease incidence can vary by geographical location of the farms, farm management practices, and herd size.

Although the cattle industry has made great improvements with herd management, animal facilities and care, feeding and nutrition, and timely use of bio-pharmaceutics, calf diarrhea is still problematic due to the multi-factorial nature of the disease. Prevention and control of calf diarrhea should be based on a good understanding of the disease complexities such as multiple pathogens, co-infection, environmental factors, and feeding and management during the calving period before disease outbreaks. In this overview, infectious agents involved in calf diarrhea, appropriate application of diagnostic methods for identifying these pathogens, and intervention strategies for managing calf diarrhea are described. The article consists of three sections. The first section presents the characteristics of major enteric pathogens known to cause calf diarrhea (*i.e.*, bovine rotavirus (BRV), bovine coronavirus (BCoV), bovine viral diarrhea virus (BVDV), *Salmonella* (*S.*) *enterica*, *Escherichia* (*E.*) *coli*, *Clostridium* (*C.*) *perfringens*, and *Cryptosporidium* (*C.*) *parvum*) along with newly emerging enteric pathogens such as bovine torovirus (BToV) and caliciviruses (bovine norovirus [BNoV] and Nebovirus). In the second section, proper sampling and handling techniques (*e.g.*, sample collection and delivery to a diagnostic laboratory) as well as various laboratory diagnostic methods are reviewed along with their

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advantages and disadvantages. The last section includes a discussion of prevention and control strategies for calf diarrhea that involve multiple factors such as peripartum calving management, calf immunity, and environmental stress and contamination.

Infectious Etiologies

Numerous infectious agents have been implicated in calf diarrhea. Bovine practitioners and cattle producers are aware of many enteric pathogens because these primary agents have been known to be involved in calf diarrhea for several decades and still greatly influence current cow-calf operations. Ten different enteric pathogens are recognized as either major (BRV, BCoV, BVDV, *Salmonella* spp, *E. coli*, *C. perfringens*, and *C. parvum*) or emerging (bovine caliciviruses and BToV) pathogens. Characteristics of different enteric pathogens (viruses, bacteria, and protozoa) including more recent findings are briefly described below.

Viruses

Bovine rotavirus is a primary etiological agent 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 (16～21 kb) and is very stable over a wide pH range with heat lability [38]. There are seven serogroups (A through G) of rotaviruses based on antigenic and genetic similarities of the intermediate capsid protein (VP6) [129]. Group A rotaviruses are the major cause of rotaviral infection in domestic animals [129]. Most BRVs (95%) belong to group A, although groups B and C rotaviruses have also been identified in field cases [45,133].

Group A rotaviruses can be further classified into P or G types based on genetic and antigenic similarities of VP4 (protease sensitive protein) and VP7 (glycoprotein) which constitute the outer capsid of the virion and induce anti-viral neutralizing antibody production [25]. Sixteen G types and 27 P types have been reported in domestic animals [25]. Bovine rotaviruses are G1, G6, G8, or G10 types [49,82]. G6 and G10 type are reported to be the most prevalent in cattle [82].

While VP4, VP6, and VP7 play a major role in maintaining viral structure, virus attachment, and antigenicity, nonstructural glycoprotein 4 (NSP4) holds a special role as a viral enterotoxin. This protein also interferes with cellular homeostasis by elevating calcium ion influx into the cytoplasm [4]. These alterations account for drastic changes in the movement of nutrients and water across the intestinal epithelium and are more important for viral pathogenesis than histopathological lesions.

Bovine rotavirus usually causes diarrhea in calves at 1 to 2 weeks of age. The milk uptaken by calves can provide a good environment for rotavirus survival under a wide range of gastrointestinal pH levels and infection of the intestinal epithelial cells [26]. This may explain why weaning calves are more susceptible to calf diarrhea. The virus has a very short incubation period (12～24 h) [129] and induces peracute diarrhea in affected calves. Once infected, the calves shed a large amount of virus via feces for 5～7 days, thus contaminating the environment and allowing the virus to be transmitted to pen mates. The virus replicates in the cytoplasm of epithelial cells of small intestinal villi. 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 maldigestive/ malabsorptive diarrhea promoted by rotavirus infection. Viral infection causes villus atrophy and usually affects the caudal part of the small intestine. Evidence for interspecies transmission along with genetic reassortment between human and animal rotaviruses (*e.g.*, swine, bovine, feline, and canine) has raised concerns about zoonotic rotaviruses [81].

Bovine coronavirus is an enveloped virus with a positive-sense, single-stranded RNA genome (27～32 kb). This pathogen is a member (*Betacoronavirus 1*) of the genus *Betacoronavirus* that was formerly classified as group 2a coronaviruses [24]. Virus infection can present as three distinct clinical syndromes in cattle: a) calf diarrhea in calves at 1 to 2 weeks of age; b) winter dysentery with hemorrhagic diarrhea in adult animals; and c) respiratory diseases including bovine respiratory disease complex in both young and adult cattle [17,77].

The spike (S) protein of the virus plays an important role in virus entry and pathogenesis besides the ability to neutralize antibody [76]. The S protein consists of two subunits (S1 and S2) and is crucial for virus-host interaction. While the S1 subunit facilitates binding of the virus to host cell receptors, the S2 subunit functions in the fusion of the viral envelope to host cellular membranes [146].

Viral infection begins in the small intestine and usually spreads through the entire small intestine and colon. Microscopically, villi of the affected small intestine and colonic crypts become atrophic, and the lamina propria becomes necrotic. Initially, the S protein and hemagglutinin-esterase (HE) protein of the virus attach and fuse to the intestinal epithelial cells. [122]. The virus replicates in enterocytes and progeny viruses are released through a normal secretory mechanism and cell lysis. Mature villous epithelial cells are the primary target of the virus although crypt enterocytes are also affected. 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 is an enveloped, positive-sense, single-stranded RNA virus (12.3 kb) and a

member of the genus *Pestivirus* in the family *Flaviviridae* [40]. There are three species included in the genus: BVDV, border disease virus, and classical swine fever virus. BVDV can be divided into two types (BVDV1 and BVDV2) based on sequence similarity of the 5' untranslated region (UTR) in the viral genome. In addition to these two types, BVDV3 was recently proposed as tentative species together with other *Pestivirus* species (*e.g.*, border disease virus type 2, Pronghorn, and Bungowannah) [46]. Each type can be further divided into two biotypes (cytopathic and noncytopathic) based on their ability to cause lytic cytopathic effects in cell culture. Noncytopathic strains of BVDV are responsible for persistent infection of the virus in cattle [52]. To date, 15 (BVDV1a to BVDV 1o) BVDV1 and two (BVDV2a and BVDV2b) BVDV2 subgenotypes have been recognized [40,63]. BVDV1a, BVDV1b, and BVDV2a are the most prevalent subgenotypes in US cattle populations [44]. BVDV1c is the most common subgenotype in Australia [118].

The clinical symptoms of BVDV infection vary from subclinical to fatal disease depending upon host immune status, pregnancy and gestation period, and the presence or absence of co-infection with other pathogens. Most infected animals develop mild clinical signs such as low-grade fever, leukopenia, anorexia, and decreased milk production. Acute BVD infection is characterized by diarrhea, pyrexia, depression, anorexia, decreased milk production, oral ulcerations, hemorrhagic syndrome, and lymphopenia/leucopenia leading to immunosuppression [2]. Immunosuppressed cattle become susceptible to other diseases due to the concurrent infection with other pathogens (*e.g.*, bovine respiratory disease complex). Although most immunocompetent animals eventually clear the virus and recover from the disease, some infected cattle occasionally harbor the virus for a long time with periodical appearance of transiently detectable viremia from time to time (*i.e.*, transiently infected animals).

Pregnant cows and heifers deliver persistently infected (PI) calves if they are exposed to a noncytopathic BVDV during 45～125 days of gestation since the fetus is not immunocompetent. Most PI calves are born weak and susceptible to other pathogens, and experience poor growth. The PI animals also develop fatal "mucosal disease" when exposed to either exogenous or endogenous cytopathic BVDV [11]. Mucosal disease is clinically characterized by mucosal ulceration, vesicle formation, erosions, diarrhea, and death. BVDV can cause calf diarrhea in two major ways: 1) persistent infection resulting in primary damage to enterocytes and susceptibility to co-infection, or 2) transient infection with replication in crypt enterocytes and lesion formation contributing to diarrhea.

Bovine torovirus is an enveloped, positive-stranded, RNA virus (25～30 kb) belonging to the genus *Torovirus* in the family of *Coronaviridae*, order *Nidovirales* [68] along with equine torovirus, porcine torovirus, and human torovirus. Toroviruses are infectious gastrointestinal agents in cattle, and a predominant cause of acute enteric infection in piglets and children [69,78]. Fecal shedding of BToVs from diarrheic calves has been reported around the world including the USA (2003, 2002, 1983, and 1982), Canada (1998), Costa Rica (1998), Korea (2008), the Netherlands (1991), Germany (1992), Hungary (2002), Austria (2006), Japan (2007), and South Africa (1993) [29,53,56,67,108,110,139]. Morphological similarities and antigenic cross-reactivity between human and bovine toroviruses has raised a concern about the potential zoonotic nature of BToV [57].

Bovine toroviruses can produce mild to moderate diarrhea in young calves less than 3 weeks of ages [57]. After oral or nasal inoculation with the virus, epithelial cells in the middle and lower parts of intestinal villi extending into the crypt epithelium are infected, leading to cell death and epithelial desquamation in the small intestine together with necrosis in the large intestine [32,112]. Damage to the villous and cryptic enterocytes thus induces malabsorptive/maldigestive diarrhea. Thirty to 50% of lesions caused by the virus are present in the upper small intestine, which may account for the mild to moderate diarrhea in affected animals [144]. Similar to BCoV, BToV antigen and viral RNA have been detected in nasal secretions, but the role of these factors in respiratory disease remains to be clarified [55].

Bovine norovirus is a non-enveloped, single-stranded positive-sense RNA virus (7.4～8.3 kb) belonging to the genus *Norovirus* in the family *Caliciviridae* [20]. Five genogroups (GI through GV) have been identified based on sequence similarities of open reading frames (ORFs) 2 (VP1: major capsid protein) and 3 (VP2: minor capsid protein) due to high genetic diversity among noroviruses (NoVs) [147]. BNoVs belong to GIII that includes two prototype strains, Jena (genotype 1; GIII-1) and Newbury 2 (genotype 2; GIII-2) viruses, and are phylogenetically distinct from human (GI, GII, and GIV), porcine (GII-11, GII-18, and GII-19) and murine (GV) NoVs [84,101,123]. The possibility of interspecies transmission of NoV was demonstrated by a study in which gnotobiotic pigs were infected with a human NoV strain, raising a concern for the zoonotic potential of this virus worldwide [16].

Numerous studies have been conducted to survey BNoV infection in cattle and molecularly characterize the viruses compared to human NoVs [19,27,64,66,85,102,106,116, 125,136,145]. The reported frequency of BNoV detection using molecular methods widely varied among different countries, ranging from 7.5% to 49.6%. All identified BNoVs have been phylogenetically distinct from human

NoVs, suggesting that the zoonotic potential of BNoVs is very low.

Noroviruses are a major cause of acute and sporadic non-bacterial gastroenteritis in humans (both adults and children). These pathogens have also been reported to cause gastroenteric disease in animals such as cattle, pigs, dogs, and mink [123]. Recently, an experimental challenge study with the Jena strain of BNoV was conducted on newborn calves infected *via* an oral route [104]. The investigators demonstrated that the virus infected epithelial cells of the small intestine and caused villous atrophy (in the jejunum and ileum) leading to diarrhea with virus shedding but not seroconversion. Detection of BNoV in feces from clinically healthy cattle has also been reported [64,91,116], raising questions about the clinical significance of BNoV.

Neboviruses belong to the newly established genus Nebovirus in the family *Caliciviridae* [14]. The viral genome is approximately 7.4 kb in length and contains two ORFs: ORF1 (encoding nonstructural proteins and capsid protein) and ORF2 (encoding small basic proteins with unknown functions) [100,124]. Newbury agent-1 and Nebraska-like bovine calicivirus form two distinct genotypes that were associated with calf diarrhea cases in the UK (1978) and Nebraska, USA (1980), respectively [100,124,143]. Since then, the presence of Nebovirus has been reported in other countries including France (2011), Italy (2011), and Korea (2008) [28,66,107]. The reported prevalence of Neboviruses in diarrheic calves ranges from 7% to 28.0% depending upon geographic location [19,28,66,100,107]. There is no evidence of zoonotic transmission. Genetic diversity has been reported to exist among Neboviruses along with identification of a novel genotype [66]. Similar to BNoVs, lesions caused by Nebovirus are observed mainly in the jejunum and ileum with villi atrophy, loss of villi enterocyte, and crypt hyperplasia when gnotobiotic calve are challenged with the virus [51,124].

Bacteria

Salmonella enterica colonizes the gastrointestinal tract of a wide range of hosts. *S. enterica* serovar Typhimurium (*S. typhimurium*) and serovar Dublin (*S. dublin*) are the most common etiologic agents that cause salmonellosis in cattle [60,127]. *S. typhimurium* is the most common serotype that affects calves in the USA [120].

Salmonella infection has a wide variety of clinical symptoms ranging from asymptomatic to clinical salmonellosis. Acute diarrheal disease is most common with *S. typhimurium* and systemic disease is associated with *S. dublin*. Calves less than 3 weeks of age are commonly infected by *Salmonella*. The lesions frequently observed in affected calves involve the pseudomembrane on the mucosa of the small intestine as well as enlargement of the mesenteric lymph nodes. Infected cattle can serve as a source of zoonosis through food-borne routes or direct contact [87].

The basic mechanism underlying *Salmonella* virulence includes the ability to invade the intestinal mucosa, multiply in lymphoid tissues, and evade host defense systems, leading to systemic disease. For *Salmonella* pathogenesis, the organism should be capable of invading intestinal epithelial cells, surviving within macrophages, and causing enteropathogenicity [132]. *Salmonella* colonizes M-cells, enterocytes, and tonsilar tissues [115]. Following lymphoid tissue (*e.g.*, tonsilar tissue) infection, *Salmonella* easily spreads throughout the whole body by invading mononuclear cells and phagocytes [58]. Salmonella pathogenicity island 1 (SPI-1) and SPI-5 are known to influence the type III secretion system, and are mainly responsible for *Salmonella*-induced diarrhea in calves [21,132]. SPI-2 is involved in the second type III secretion system and is responsible for intracellular survival of the organism [97].

Clinical presentation of salmonellosis is characterized by watery and mucoid diarrhea with the presence of fibrin and blood [41]. 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 [41]. Calves can shed the organism for variable periods of time and intermittently depending on the degree of infection (*e.g.*, clinical or subclinical infection).

Escherichia coli can be classified into six pathogroups based on virulence scheme: enterotoxigenic *E. coli* (ETEC), shiga toxin-producing *E. coli*, enteropathogenic *E. coli*, enteroinvasive *E. coli*, enteroaggresive *E. coli*, and enterohaemorrhagic *E. coli* [65,95]. Among these bacteria, the most common cause of neonatal diarrhea is ETEC stains that produce the K99 (F5) adhesion antigen (commonly referred to as *E. coli* $K99^+$) and heat-stable enterotoxin [95]. It should be noted that other pathogroups of *E. coli*, which are usually identified by histopathology, can be missed if the diagnosis focuses on *E. coli* $K99^+$ alone.

Neonatal calves are most susceptible to ETEC infection during first 4 days after birth and develop watery diarrhea if infected [42]. Following ingestion, ETEC infects the gut epithelium and multiplies in enterocytes of the intestinal villi. The distal portion of the small intestine provides the most favorable environment for ETEC colonization due to the low pH (less than 6.5). Villous atrophy due to a loss of infected cells and damage to the laminar propria are commonly observed in affected small intestine. The bacteria express the K99 antigen for attachment [43]. After colonization of the gut epithelium, heat-stable toxin

production induced by ETEC leads to the up-regulation of chloride secretion into the gut. This osmotically pulls water into the intestinal lumen and leads to the development of secretory diarrhea in calves.

Clostridium perfringens is a Gram-positive, spore-forming anaerobic bacterium that causes a wide range of diseases in mammals and birds [137]. These microorganisms can be subdivided into five toxin types (A, B, C, D, and E) based on the production of four major toxins: alpha (α), beta (β), epsilon (ε), and iota (ι) [111]. Type A strains produce α toxin alone, type B stains produce α, β, and ε toxins; type C type strains manufacture α and β toxins; type D strains secrete α and ε toxins; and type E strains produce α and ι toxins. Among these groups, type C has been frequently reported in conjunction with calf diarrhea [119] but not as common as some other enteric pathogens such as BRV, BCoV, *E. coli*, *Salmonella* spp., and *C. parvum*.

The α toxin is the main lethal toxin and promotes cell lysis through the hydrolysis of membrane phospholipids [110,128]. The β toxin is highly trypsin-sensitive and induces mucosal necrosis [111]. The ε toxin causes lethal enterotoxemia in domestic animals, and the ι toxin is responsible for dermonecrosis due to its high vascular permeability [111]. Enterotoxin causes diarrhea and intestinal cramping due to its effects on epithelial tight junction protein [86]. Beta-2 toxin, which is produced by all types of *C. perfringens*, has been recently postulated to synergistically function with enterotoxin [50].

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 produce a low level of proteolytic enzymes (*e.g.*, trypsin) in the gastrointestinal tract can be easily infected by *C. perfringens* type C since β toxin is recognized as the main virulence factor responsible for clinical signs seen in animals affected by this bacterium. Intestinal lesions in these infected animals are characterized by diffuse or multifocal hemorrhagic necrotizing enteritis and bloody fluid distension [6].

Protozoa

Cryptosporidium parvum is a protozoan parasite that is frequently associated with gastrointestinal tract disease in humans and neonatal cattle. Calves infected with *C. parvum* can be asymptomatic or develop severe diarrhea with dehydration [35,36]. There are approximately 24 species of *Cryptosporidium* [34]. Cattle are commonly infected by *C. parvum*, *C. bovis*, *C. ryanae*, and *C. andersoni*. *C. parvum* is considered to be primary cause of calf diarrhea and is a potential zoonotic agent [15].

Once *C. parvum* is ingested, the oocyst excystation releases sporozoites that penetrate enterocytes. The excysted parasites undergo asexual (type I meront) and sexual (type II meront) reproduction to produce macrogametocytes and microgametocytes. Upon fertilization of the macrogametocytes by microgametes, zygotes are developed with sporulates (*sporogony*) generating thin-walled oocysts involved in autoinfection. Next, thick-walled oocysts pass out of the host. The oocysts can survive for more than a month in the environment under favorable conditions (*e.g.*, high temperature and moisture with low UV radiation) and are resistant to most disinfectants [37]. Environments contaminated with oocysts can be an immediate source of infection for both animals and humans.

The invasion of *C. parvum* into enterocytes induces changes in intestinal cytoskeleton structures, such as loss of microvilli and shortening of columnar epithelial cells, leading to severe villous atrophy in infected animal [54]. Damage to the intestinal epithelium causes prolonged malnutrition and reduced growth rates in affected calves due to malabsorption and fermentation of undigested milk in the intestinal lumen [96]. These result in considerable economic losses in cow-calf production.

Diagnosis of Calf Enteric Pathogens

Diarrhea can be fatal to neonatal calves due to dehydration and acidosis that may result in anorexia and ataxia [10]. Since various pathogens or factors have been implicated in the development of diarrheic disease, laboratory testing is necessary for accurate assessment of the problem (*i.e.*, accurate diagnosis). The progression of diarrhea can be rapid. Hence, a quick diagnosis is critical for not only quickly confirming the cause but also helping clinicians and cattle producers to implement appropriate interventions in a timely manner. It should be noted that the diagnostic outcomes can be influenced by many factors such as sampling time and population, types and quality of the specimens, and laboratory methods used. Each of these factors is discussed below.

Procedures for diagnosing calf diarrhea

Clinical (*e.g.*, age, vaccination record, and clinical signs) and farm history should be provided to clinicians for determining the cause of diarrhea. Once the specimens are submitted to a veterinary diagnostic laboratory, the diagnostician sorts the samples to ensure proper delivery to testing laboratories based on the history and sample type. Generally, fecal sample are examined by microscopy (for *C. parvum* and Coccidia), bacterial culturing (for *Salmonella* spp., *E. coli*, and *C. perfringens*), and PCR (for BRV and BCoV). In contrast, intestinal tissues are subjected to immunohistochemistry or bacterial culturing. More recently, nucleic acid-based techniques such as PCR and an antigen-capturing enzyme-linked immunosorbent

assay (Ag-ELISA) have been more commonly used for the rapid detection of various bacterial and viral pathogens in clinical specimens from diarrheic calves. When the laboratory test results are available, clinicians should consider the overall farm and clinical history in conjunction with lab results before identifying the causative pathogen.

Sampling and specimen submission

Proper specimen collection and delivery to a diagnostic lab is commonly neglected, and 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 sacrificed, moribund, or euthanized calves are of great value for diagnosis during severe outbreaks. Fresh and formalin-fixed gastrointestinal tissues (abomasum, small intestine, or colon) including ones from regional lymph nodes and liver should be collected along with colonic contents. Fresh fecal samples should be directly recovered from diarrheic animal into a specimen container with either rectal swabs or by rectal stimulation while avoiding environmental contamination (by soil, urine, or other feces). Once collected, the sample should be stored in a transporting medium or special stool container with refrigeration to maintain pathogen viability and sample integrity (*i.e.*, reduced overgrowth of undesired bacteria and prevention of nucleic acid degradation) [75]. Samples of anaerobic bacteria (*e.g.*, *C. perfringens*) should be kept in an oxygen-free transport medium during shipping if possible.

Laboratory testing

Laboratory methods for identifying enteric pathogens have typically included pathogen isolation and characterization along with histopathology as the gold standard for etiologic agent and disease confirmation [114]. However, many enteric pathogens are difficult to isolate from the gastrointestinal environment [31]. Direct visualization (*e.g.*, light microscopy or electron microscopy [EM]) of pathogens in feces or intestinal contents as well as the detection of antigens (*e.g.*, Ag-ELISA) or nucleic acids (*e.g.*, PCR) in specimens have been widely accepted as alternative methods. Most veterinary diagnostic laboratories concurrently use numerous techniques when testing samples for enteric pathogens. The characteristics along with advantages and disadvantages of common laboratory methods for identifying enteric pathogens are briefly described below and summarized in Table 1.

The virus isolation test is still considered the 'gold standard' for detecting viral pathogens in specimens [114] although new methods such as an ELISA and PCR-based tests have been developed. Cell culture techniques are commonly used to isolate virus for diagnostic purposes as well as virus propagation for vaccine production or further virus characterization procedures such as antigenic variation determination or gene sequencing [117]. Several cell lines (*e.g.*, Madin Darby Bovine Kidney [MDBK], human rectal tumor HRT-18, and African green monkey kidney MA104 cells) are used for certain viruses due to variations in viral susceptibility of the different cells [1,140]. Embryonating eggs and laboratory animals are also used for isolating and propagating viruses which do not grow in cells *in vitro* (this is the case for many enteric pathogens) or increasing viral production. The viability of target viruses in a specimen is critical for successful virus isolation [121]. Specimens should be kept at a low temperature and in transport medium during shipping to a diagnostic laboratory and delivered to the lab as soon as possible after collection. The virus isolation test is a confirmatory method; however, it takes a time to prepare the cells and propagate the virus (*i.e.*, slow turnaround of the results). This technique is therefore laborious and expensive compared to an ELISA or PCR.

Electron microscopy is commonly used for virus detection and identification based on morphological characteristics. There are two types of EM: direct EM and immuno-electron microscopy (IEM) [12]. Two different staining techniques (positive and negative staining) are performed to visualize the target. For direct EM, virus particles in a fluidal sample matrix are applied directly to a solid support and then visualized after a contrast stain is applied. This procedure is commonly referred to as "negative staining EM" whereas positive staining is generally used for thin-section EM of fixed tissues. Direct EM is not a specific test as this technique is performed to simply visualize viruses in samples and is not considered to be a sensitive procedure. In comparison, IEM has greater sensitivity than direct EM since the specimen is incubated with antibody specific for the target virus in order to agglutinate the virus before staining.

The visualization of viruses, particularly ones not amenable to cultivation, is a major advantage for EM with rapid turnaround. Most bovine enteric viruses such as BNoV, Nebovirus, BRV, BToV, and BCoV are difficult to isolate or propagate in cell cultures, but these pathogens can be differentiated according to their unique morphology (both shape and size) under an electron microscope [29]. EM requires a large number of virus particles (approximately $10^4 \sim 10^6$ virus particles per mL) in the specimen for virus detection (*i.e.*, low sensitivity) and cannot concurrently evaluate multiple samples [38]. The collection of fecal samples from clinically ill animals with acute diarrhea is important for successful EM. The cost of electron microscopes and requirement of skilled laboratory personnel is still a challenge for EM use as a routine

Table 1. Advantages and disadvantages of laboratory methods for identifying enteric pathogens

Diagnostic method	Advantages	Disadvantages	Target pathogens
Virus isolation	- Confirmation of the presence of infectious virus in clinical specimens - Availability of isolated virus for further characterization or vaccine production - Lack of specificity	- Low sensitivity - Restriction by characteristics of cells used for viral production - Requirement of proper sample collection and handling for virus viability - Not applicable for cytotoxic specimens - Time-consuming and laborious	BRV, BCoV, BVDV
Electron microscopy	- Applicable for non-cultivatable virus - Morphological visualization - Lack of specificity	- Requires a large number of virus particles in the samples - Low throughput - Need for skilled personnel - Expensive instrumentation	BRV, BCoV, BVDV, BToV, BNoV, Nebovirus
Antigen-capturing enzyme-linked immunosorbent assay	- Rapid detection of pathogens - High-throughput testing - Plug-in-and-play capability - Portability	- Low analytical sensitivity - Cost-prohibitive in some situation - Specificity problemsdue to non-specific binding or background signals	BRV, BCoV, *E. coli*, K99^{+}, *C. perfringens*, *C. parvum*, BVDV, BToV, BNoV, Nebovirus
Fecal flotation and direct microscopy	- Commonly used for parasite eggs or oocysts - Rapid detection - Low cost	- Low sensitivity - Requires an optimum number of oocysts - Subjective interpretation of results	*C. parvum*
Fecal bacteria culture	- Commonly used for bacterial pathogens identification - Lack of specificity	- Slow turnaround time - Requires the presence of infectious bacteria - Laborious	*Salmonella* spp, *E. coli*, K99^{+}, *C. perfringens*
Latex agglutination test	- Wide range of targets - Semi-quantification capabilities - Cheap procedure with rapid turnaround	- False positive results due to non-specific binding - Low analytic sensitivity	*E. coli*, K99^{+}
Conventional PCR	- Rapid detection of pathogens - High sensitivity and specificity	- Experienced personnel required - Risk of contamination during sample processing - False negative results due to genetic mutation or recombination - Low throughput	BRV, BCoV, BVDV, BToV, BNoV, Nebovirus, *Salmonella* spp, *E. coli*, K99^{+}, *C. perfringens*, *C. parvum*
Real-time PCR	- Rapid detection of pathogens - High throughput - High sensitivity and specificity - Quantification of target pathogen	- High cost - Limit of PCR product size - Cross-talk between different dyes - False negative results due to genetic mutation or assay inhibition - False positive results due to cross-contamination	BRV, BCoV, BVDV, BToV, BNoV, Nebovirus, *Salmonella* spp, *E. coli*, K99^{+}, *C. perfringens*, *C. parvum*

BRV: bovine rotavirus, BCoV: bovine coronavirus, BVDV: bovine viral diarrhea virus, BNoV: bovine norovirus, BToV: bovine torovirus, *C. parvum*: *cryptosporidium parvum*, *C. perfringens*: *clostridium perfringens*.

diagnostic test. Nevertheless, EM is a tool to use when diarrhea with an unknown infectious cause is encountered.

An antigen-capturing ELISA is performed for rapidly detecting a pathogen in a clinical specimen based on antibody (*e.g*., monoclonal antibody) recognition of the target antigen [74]. For this method, antibody is attached to a solid surface such as glass, plastic, or a membrane filter. The antibody captures target antigen present in the sample. A cascade of colorimetric reactions then verifies antigen capture and indicates an antigen-antibody reaction. Antigen concentration can be quantitatively estimated as optical density (OD) measured by spectrometry.

The Ag-ELISA has been utilized in many fields. In particular, this method has been extensively performed in human diagnostic medicine. There are several platforms being used including the tube method, microtiter plate method, and membrane-bound method [38]. While the microtiter plate method has been commonly employed in diagnostic laboratory settings, the membrane-bound method using a lateral flow technique, such as a strip test, SNAP test, or rapid kits, is the most common platform for in-clinic or patient-side tests. Commercial Ag-ELISA kits for detecting BRV-A, BCoV, *E. coli* $K99^+$, and/or *C. parvum* in fecal samples are available. Ag-ELISAs are well known for rapid turnaround, high-throughput testing, plug-in-and-play capability, and portability [38]. Analytic sensitivity of this method tends to be lower than that of isolation/culture or nucleic-acid based assays [18]; therefore, collection of samples from animals with acute diarrhea is important for reliable test results. For the best data, feces should be freshly collected from acutely affected calves. In some situations, the expense of a commercial kit may be cost-prohibitive.

Fecal flotation and direct microscopy are commonly used to diagnose parasite eggs or oocysts. The principal of fecal flotation is simply based on the density difference between a flotation solution (≥ 1.24) and oocysts ($1.05 \sim 1.24$) [5]. A centrifugation step is commonly included in the testing procedure to increase detection sensitivity since centrifugation concentrates the target for easy viewing under a microscope. Direct microscopy can also be performed for fecal smears without centrifugation.

Oocysts in clinical specimens may be difficult to visualize without special staining. *C. parvum* oocysts are reported to be positive for acid-fast staining [93]. Modified acid-fast stains are applied to fecal smears to detect these organisms. Unlike the Ziehl-Neelsen modified acid-fast stain, the modified Kinyoun acid-fast stain contains a more concentrated fuchsin dye and lipid solvent, and does not require heating the reagents used for staining [79,131]. In brief, one to two drops of feces is smeared on a clean glass slide and air-dried. The sample is fixed with absolute methanol, and subsequently stained with carbol fuchsin and 1% sulfuric acid. The specimen is then counterstained with methylene blue or brilliant green and examined under a light microscope with oil immersion. The red or purple stained *C. parvum* oocysts 4 to 6 μm in diameter should appear against a blue or green background. This modified acid-fast staining method is widely used to detect *C. parvum* in feces. The sensitivity of this technique is low because the procedure requires approximately 500,000 oocysts per 1 g of feces to confirm the presence of *C. parvum* oocysts [3].

Fecal bacteria culturing is a commonly used laboratory method for isolating and identifying bacterial pathogens in feces and intestinal contents. *Salmonella* spp., *E. coli* $K99^+$, and *C. perfringens* are primary bovine enteric pathogens [39,62]. In order to prevent any cross- contamination or loss of viability, feces should be collected directly from diarrheic calves by either rectal swabs or rectal stimulation. Once collected, the fecal samples should be stored in a transport medium or special stool container in a cooler or on ice before submission to a diagnostic lab. To examine anaerobic bacteria-like *C. perfringens*, fecal samples must be immediately stored in a pre-reduced (*i.e*., oxygen-free) transport medium if available.

Blood agar plates, MacConkey agar plates, MacConkey agar with sorbitol, Hektoen enteric (HE) plates, and xylose lysine desoxycholate (XLD) plates are used for bacterial culture [23,99]. Several kinds of enriched and selective media such as brain heart infusion (BHI) broth (a highly nutritious medium for general bacterial culture) and tetrathionate broth (for *Salmonella* spp.) are employed for growing and identifying certain bacterial pathogens. Blood agar is most commonly used because the majority of bacteria can grow on this medium. MacConkey agar is selectively used to culture Gram-negative bacilli that are commonly present in the gastrointestinal tract and differentiate bacteria that ferment lactose. Sorbitol-MacConkey agar can help distinguish nonpathogenic *E. coli* from *E. coli* O157:H7 which cannot ferment sorbitol [33]. *Salmonella* spp. are typically cultured from fecal samples using Samonell-Shigella agar, bismuth sulfite agar, HE medium, brilliant green agar, and XLD agar [138]. For *C. perfringens* culturing, thioglycolate broth growth medium is commonly used. Culturing usually takes 2 days at 36^oC under anaerobic conditions [39]. Colony morphology (*e.g*., shape, surface, and elevation of colonies on the agar plates), physical characteristics of the bacteria (*e.g*., aerobe, anaerobe, or microaerophile), microscopic features (*e.g*., rods, cocci, or coccobacilli), and biochemical tests (*e.g*., ones that 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 results (growth and

identification can take 24～72 h) is a disadvantage of bacterial culture tests although the turnaround can vary depending on culture methods and diagnostic instrumentation. In some cases, further immunological testing (*e.g.*, an agglutination test) is required for the identification (*e.g.*, for *E. coli* K99$^+$) [18] or serotyping (*e.g.*, for *Salmonella* spp.) of bacteria [73]. A nucleic acid-based assay is also required for typing (*e.g.*, for *C. perfringens* toxin type) [50].

The latex agglutination test (LAT) is in principle similar to an ELISA [113]. The surface of latex particles is coated with antigen or antibody. The particles can then capture antibody or the target antigen, respectively. This test has been applied to detect a wide range of targets such as bacteria, virus, hormones, drugs, and serum protein [105].

Latex particles are made of synthetic rubber and emulsified as billions of micelles of the same size with a desired diameter. Particle size typically ranges between 0.05 to 2 μm in diameter, and the presence of sulfate ions provides an inherent negative surface charge to the particles [109]. The prepared latex particles can be further functionalized by special processes such as amidation, amination, carboxyation, hydroxylation, or magnetization to increase their binding stability and analyte attachment depending upon the purpose of the test [109].

For cases of calf diarrhea, the LAT has been frequently performed to identify *E. coli* K99$^+$ [18]. Fecal samples are collected from diarrheic calves and sent to a diagnostic lab for evaluation. Once the *E. coli* is isolated, the bacterial suspension is mixed with latex beads coated with anti-*E coli* K99$^+$ antibody and incubated under specific conditions. Agglutination of the latex beads can be clearly visualized when K99 antigen is present in the isolated *E. coli*. The latex agglutination test is frequently employed in diagnostic labs because this method can serve as a semi-quantified test and is relatively cheap with rapid turnaround [47]. Caution should be taken when interpreting marginal results since false positive/negative results frequently occur due to non-specific binding or interference [113].

Polymerase Chain Reaction (*PCR*) is a common nucleic acid-based method for detecting enteric pathogens. PCR involves thermocyclic enzymatic amplification of specific DNA sequences of the target pathogen using a pair of oligonucleotide primers that hybridize to DNA/cDNA regions of interest in the genomic sequence. Genomic material of the target pathogen is first extracted. Next, the sample is mixed with a heat-stable DNA polymerase (*e.g.*, Taq DNA polymerase), dNTPs, primers, and PCR buffer. DNA amplification usually proceeds for 25 to 40 cycles in an automated thermal cycler [30]. Each cycle includes a double-stranded DNA denaturation step, primer annealing to 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 after electrophoresis and staining with ethidium bromide that binds to double-stranded DNA. Successful amplification of the target sequence is determined based on molecular size and/or sequencing of the PCR product.

PCR testing is especially useful for detecting viruses that are difficult to isolate in cell culture or bacteria that require a long time to grow [31]. There are numerous commercial PCR reagents available which provide convenience, high sensitivity, and rapid results. PCR testing requires trained and experienced technicians. Inadvertent contamination during sampling in the field or processing at the laboratory can be a source of false positive results due to its high sensitivity. Viruses with a high mutation rate, often RNA viruses (*e.g.*, rotavirus and calicivirus), need to be continuously monitored for sequence changes in the target gene otherwise negative results will be obtained due to primer incompatibility. Fecal samples are known to contain factors that inhibit PCR and can lead to false negative results if appropriate reagents or steps to remove such inhibitory substances are not included in the test procedures.

Real-time PCR (as known as quantitative PCR or qPCR) is a method that is capable of not only amplifying the target sequence but also quantifying the amount of target with great sensitivity and high throughput [142]. There are three types of real-time PCR methods commonly used for diagnostic purposes: TaqMan, molecular beacon, and SYBR Green real-time PCR. TaqMan real-time PCR involves a oligonucleotide probe labeled with two types of fluorophores (*i.e.*, a report dye and quencher dye) in addition to a primer pair [31]. The reporter dye is located on the 5′-end of the probe and the quencher is attached to the 3′-end. After denaturation of the DNA template, the primers and probe bind to each strand of the template. Extended primers remove the TaqMan probe from the template DNA, and the reporter dye is thus separated from the quencher dye. Emission from the reporter dye (*e.g.*, fluorescence energy) can be detected spectrophotometrically. All real-time PCR steps are conducted in a closed tube system; hence, the opportunity for contamination can be minimized. The assay provides high specificity due to detection of probe signal based on primer extension. Real-time PCR using a molecular beacon probe is similar to TaqMan real-time PCR. However, the beacon probes form a hair-shape structure since the probe sequence is placed between the "arm" sequences and produce bright fluorescence when bound to their target template [80].

The principle of SYBR Green real-time PCR is based on SYBR Green dye binding to double-stranded DNA that will produces light when excited. SYBR Green assays are

cheaper than TaqMan real-time PCR techniques. However, the dye binds to any double-stranded DNA molecule. Therefore, SYBR Green real-time PCR requires a melting-curve analysis to determine whether the amplification curve is produced by the intended target or other factors such as primer dimers or non-specific amplicons [59].

There are several kinds of reporting dyes used for probe-based real-time PCR assays based on fluorescence energy wavelength. This facilitates multiplexing by combining different reporting dyes. Theoretically, multiplex real-time PCR can simultaneously detect up to four different targets in the same sample [18]. Nevertheless, there is a size limit for the PCR product (usually less than 200 bp) in order to maintain stable sensitivity [141]. The primers and probes should thus be carefully designed when a multiplex real-time PCR assay is performed. "Cross-talk" between different dyes due to close proximity in fluorescence energy wavelength is another factor to take into consideration when multiplexing.

Prevention and Control of Calf Diarrhea

Calf diarrhea is a multifactorial disease [62,134]. Factors involved in the occurrence of calf diarrhea can be summarized as ones associated with a) peripartum calving management, b) calf immunity, and c) environmental stress or contamination. Characteristics of major or emerging bovine enteric pathogens were previously described in this review. There is not much of difference between the patterns of disease development and prevention of calf diarrhea according to each etiological agent. Knowing of causal pathogen(s) is important for accurately assessing the current status of the affected farm and developing further interventions. Nowadays, disease control and prevention in production animals involves animal welfare from the public or consumer's point of view, and increased productivity from the 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. Inadequate feed intake and macro- or micro-nutrient deficiencies during the last trimester increase calf morbidity and mortality rates because most fetal growth occurs during last 2 months of gestation [89,90]. The quality and quantity of colostrum is associated with body condition score (BCS). A BCS near 5 (on a scale of 1 ~ 10) is acceptable for multiparous cows and a score of 6 for primiparous cows at calving is desirable [70]. Recently, cow nutrition has been shown to impact the transition of the calf into adult life as well as fetal growth and development [48]. Calves born to underfed cows have poor growth performance, low productivity, and higher susceptibility to disease. In another study, heifer calves born to cows fed supplemental protein during the last trimester were found to have greater pregnancy performance later in life compared to the control group [83].

Dystocia is closely related to poor calf performance as well as increased susceptibility to environmental pathogens which frequently cause calf diarrhea [71]. Calves that experience dystocia may have physical symptoms such as congestion and swelling of the head and tongue, which can reduce the amount of colostrum uptake from the dam. The absorption rate of colostrum-derived immunoglobulin is lower in these calves compared to healthy animals [98]. Consequently, the affected calves cannot obtain appropriate passive immunity from the dams due to inadequate colostrum uptake during early life (*i.e.*, 2~ 6 h after birth) [92].

The major causes of dystocia are associated with large calf size and small pelvic size of the dam. Large calves are more likely to have an improper position and presentation (*e.g.*, backward, breech, and mal-positioned limbs or head) in the uterus. Under these conditions, the head and legs cannot enter the birth canal. Insufficient maternal pelvic size also can induce dystocia, especially in beef heifers. To prevent dystocia, the dam's genetic inheritance (*e.g.*, adequate pelvic size and calving ease) should be taken into consideration during heifer selection [9], and frequent monitoring of the calving cow is required for appropriate calving assistance [71].

Immunity

The bovine placenta does not permit the passive transfer of antibody to the fetus. As a result, the newborn calf does not receive any antibody from the dam and is very susceptible to environmental pathogens. Resistance of the calf to enteric disease is closely related to the timely consumption of high-quality colostrum in sufficient quantities [7]. The neonatal calf should ideally receive 2 ~ 3 L (for beef calves) or 3 ~ 4 L (in dairy calves) of colostrum within the first 6 h after birth [22]. The colostrum contains 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) [94]. Immunoglobulin G is the primary antibody isotype in bovine colostrum.

The quality of colostrum varies based on calving number, nutritional status, and vaccination of the cow [98]. However, calves born to heifers can receive an acceptable level of maternally derived immunity if enough volume of colostrum is ingested within the first 24 h of life [71]. Heifers have a greater likelihood for dystocia, mis-mothering, and poor colostrum production compared

to a multiparous cow. Therefore, cow-calf management practices (*e.g.*, calving heifers first and segregation of calves based on birth date) should be considered for reducing the chances of infectious disease development.

The primary function of colostrum is to enhance the calf's immune system through the passive transfer of both antibody and cell-mediated immunity. Ideally, calves should receive colostrum from their dams although colostrum from several cows is often mixed and administered or purchased. One caution of colostrum feeding is the transmission of BVDV, bovine leukemia virus, and Johne's disease that can be spread by infected or purchased colostrum [88,130]. In particular, *Mycobacterium avium paratuberculosis* (Johne's disease) transmission is the number one risk factor associated with colostrum acquired from dairy cattle and administered to beef cattle. Therefore, colostrum from dairy farms of unknown infection status should be avoided. It is recommended that supplemental colostrum should be obtained from the farm of origin or a historically disease-free facility.

If animals on a farm have been suffering from specific pathogens such as BRV, BCoV, *C. perfringens*, and *E. coli* $K99^{+}$, vaccination of the dams could increase the concentration of specific antibodies against targeted pathogens in the colostrum [22]. Currently, commercial multivalent vaccines for these pathogens are available. Most vaccines contain either live modified or killed organisms, or a combination of the two. Some vaccines are specific for cows while the others are designed for calves.

Environmental stress and contamination

Harsh weather conditions such as low temperatures, rain, heavy snow, wind, and high levels of moisture act as stress factors to young calves and increase the susceptibility of calves to diarrhea [13,71]. Neonatal calves are not able to effectively regulate their body temperature when exposed to extreme weather conditions. This may induce hypothermia or hyperthermia resulting in immune system impairment. The dam is less influenced by environmental stress than the calf. However, the probability of dystocia or metabolic disease is still increased by environmental stress [89]. Special care is required to reduce environmental risk factors closely associated with calving season including the provision of dry, draft-free shelter. The calving season can be adjusted to a time when environmental conditions are more favorable by implementing a controlled breeding program.

Exposure to a contaminated environment is the main cause of calf diarrhea. A simple solution would be to reduce the pathogen load into the environment where calves are raised although this has always been a challenge for cattle 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 a lack of calf segregation by age [71,72]. These factors usually work synergistically and increase the opportunity for increased duration of exposure to a higher quantity of pathogens. Conversely, intervention for preventing calf diarrhea is focused on the control and reduction of each factor (*e.g.*, pathogen load and environment contamination). The basic concepts of intervention for reducing the incidence of calf diarrhea are based on 1) decreasing pathogen exposure by planning to breed and heifers first calving, which reduces the exposure of more susceptible newborn calves to pathogens, 2) reducing pathogen loading into the environment by shortening the calving season through scheduling breeding, which reduces the period of pathogen entry into the environment; and 3) keeping a clean area (or pathogen-free area) by grouping animals according to their calving date so that the calving area can be kept clean after occupation by the previous calving group.

The Sandhills Calving System has been reported to be highly effective for controlling calf diarrhea caused by multiple pathogens [126]. The system is based on preventing pathogen exposure during the early stage by segregating groups of calves in the order of calving time and maintaining a clean calving area. Essentially, a group of cows is moved into the first calving pasture when calving begins and calving continues in the first pasture for 2 weeks. Cows that have not yet calved by the end of the second week are moved to a second pasture where calving continues for 1 week. Any remaining cows that have not calved are moved to the third pasture where calving continues for another week. Finally, calves born in different pastures are grouped together when the youngest calves are 4 weeks old. The calving interval in each pasture area can vary depending upon herd size, available pasture, and previous history of calf diarrhea for each farm.

Although the Sandhills Calving System was initially introduced for pasturing calving cows, the concept is applicable for dry lot calving depending upon the situation of each farm. For example, when the pasture area is not large enough for rotational calving or cow-calf segregation, a corn or soybean field can be utilized as a calving lot or for isolating sick animals in lieu of a pasture area during the off-season (*e.g.*, after harvesting or before crop cultivation). The principle of the Sandhills Calving System for preventing calf diarrhea can be applied to dairy cow-calf operations. The calves must be immediately moved from the calving pen to an individual pen or hutch after birth to avoid contamination with pathogens. The colostrum must be immediately fed to the calf with a milking bottle rather than directly nursing from the dam. The calf pens or hutches need to be sanitized and packed with dry bed due to immune impairment of the newborn calves. The calves must be

separated from each other with enough air space to prevent contact and contamination from feces and urine of other calves. Finally, all feeding facilities and equipment (milking bottles and water buckets) should be maintained with strict hygiene practices.

Conclusion

Calf diarrhea has been a major disease that negatively affects the cattle industry. The economic impact caused by this condition is significant although many new intervention strategies (*e.g.*, vaccine, medications, and herd management) have been developed and implemented to minimize the economic loss. Persistence of this significant problem in the field may be attributed to the multifactorial nature of calf diarrhea including permutations of infectious diseases, a failure to clearly understand the disease ecology, poor environmental hygiene, and biased epidemiological data. Genetic diversity, continuous evolution, emerging pathogens, and/or environmental ubiquity of pathogens are factors that hinder effective control of the disease. Therefore, the genetic evolution of RNA viral pathogens such as BRV, BCoV, BVDV, BToV, BNoV, and Nebovirus should be kept in mind and monitored with regular genomic sequence updates. Non-group A BRV might be considered for future studies to increase the detection range of calf enteric pathogens. Emerging viruses should be regularly monitored for the evaluation of vaccines against calf enteric pathogens. Clinical significance of caliciviruses (BNoV and Nebovirus) must be carefully assessed to better control calf diarrhea in the future.

The use of highly sensitive diagnostic tests has increased the detection frequency of pathogens that were previously neglected. Therefore, optimized and appropriate diagnostic methods or platforms should be employed for detecting target pathogens in an accurate and timely manner with a minimum testing outcome bias. Currently, real-time PCR-based techniques are widely implemented in many veterinary diagnostic laboratories. These methods are highly accurate and provide high throughput performance but sometimes might overestimate the significance of pathogens detected in cases of calf diarrhea. The pros and cons of diagnostic test results and their overall interpretation must therefore be cautiously evaluated by referring clinical history from practitioner when the causative etiology is being determined.

Non-infectious risk factors have frequently been neglected by cattle producers, and also be considered equally important as infectious factors because the newborn animals are vulnerable to environmental stresses. The management and control of calf diarrhea before an outbreak is more cost-efficient than treating sick animals after the outbreak occurs. Although many enteric pathogens are involved in calf diarrhea, infection and transmission is accomplished *via* a fecal-oral route. Care must be thus taken to prevent pathogen transmission. Advice from professional consultants such as veterinarians and nutritionists is necessary to obtain an accurate diagnosis and control or manage risk factors associated with calf diarrhea in modernized large production systems.

In summary, the effective control of calf diarrhea should be based on three major points. First, a clear understanding of pathogen characteristics (*e.g.*, mechanism underlying pathogenicity, prevalence in the field, and genetic evolution) is required. Second, advantages and disadvantages of various diagnostic methods and their application to diagnostic investigation along with clinical history should be considered. Finally, proper cow-calf management is necessary for disease prevention and control.

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