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Clinicopathological Changes and Infectious Pathogens in Korean Indigenous Calves with Diarrhea

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Abstract

Clinicopathological Changes and Infectious Pathogens in Korean Indigenous Calves with Diarrhea

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Calf diarrhea is a multifactorial disease that leads to the loss of body fluid through the intestine. It has affected the morbidity and mortality of neonatal calves, impacted their growth performances, and caused worldwide economic loss in cattle

industries. Despite comprehensive investigations into the origin, prevention strategies, and treatment methods for calf diarrhea, the disease remains a pervasive issue causing substantial losses worldwide. Therefore, the purpose of this study was to deepen our understanding of calf diarrhea, especially in Korean indigenous calves (KIC) in the ROK, aiming to reduce the damages from diseases.

Specifically, the first study was conducted to identify the useful blood parameters in diagnosing calf diarrhea in KIC and good indicators for calf diarrhea. In 530 KIC, fecal scores were recorded on a scale of 0 to 3, and blood samples were collected and analyzed for hematology and serum biochemistry. Among the blood variables, 16 blood variables showed significant differences ($p < 0.01$) according to fecal scores. After reference intervals of these 16 blood variables were calculated, the distributions of calves by calculated reference intervals showed a significant difference ($p < 0.001$) and linear associations ($p < 0.001$) in blood urea nitrogen (BUN), glucose (GLU), blood sodium concentration (Na), blood potassium concentration (K), fibrinogen (Fib), and haptoglobin (Hp). Of 6 blood variables, the optimal cut-off values were calculated for BUN, K, Fib, and Hp, and the area under the curve was 0.5 or more: BUN (9.5 mg/dL, AUC: 0.623), K (5.8 mmol/L, AUC: 0.599), Fib (650.0 mg/dL, AUC: 0.706), and Hp (12.5 mg/dL, AUC: 0.847). The findings of this study not only facilitates early and accurate diagnosis of calf diarrhea but could potentially guide personalized treatment approaches by indicating the severity of the disease. This could in turn enhance the prognosis and reduce the morbidity associated with calf diarrhea.

The second study was conducted to investigate the prevalence of seven pathogens causing calf diarrhea: bovine rotavirus (BRV), bovine coronavirus (BCV), *C. parvum*, bovine viral diarrhea virus (BVDV), *Eimeria* species, *E. coli* K99, and *Salmonella* spp. in 2016 ~ 2017. A total of 544 feces from KIC in 2016~2017 were obtained to investigate selected seven pathogens causing calf diarrhea. Among 544 feces collected in 2016~2017, the number of feces that scored 0, 1, 2, and 3 were 153, 187, 116, and 88, respectively. In this study, fecal scores were significantly higher in summer and pathogens-involved feces ($p < 0.05$) and the number of detected pathogens ($p < 0.01$). Among 7 pathogens, the detection rates and mean fecal scores for each were as follows: *Eimeria* spp. (27.4%, 1.36), BRV (8.8%, 1.85), BCV (8.5%, 1.36), *C. parvum* (4.4%, 1.91), BVDV (0.7%, 0.50), and *E. coli* K99 (0.2%, 3.00). *Salmonella* spp. was not detected in any of the 544 fecal samples from KIC. The detection rates of BRV ($p < 0.001$) and *C. parvum* ($p < 0.01$) were getting increased as fecal scores were increased. Moreover, BCV showed a significant association of concurrent infection with *C. parvum* ($p < 0.01$) and BRV ($p < 0.05$). These results will be fundamental to understanding the host–agent ecology and dynamics of the pathogens in diarrhea in KIC and to developing effective prevention strategies including vaccine development.

The third study was conducted to investigate the prevalence of 7 pathogens causing calf diarrhea (BRV, BCV, BVDV type 1 and 2, *C. parvum*, *Giardia* spp., and *Eimeria* spp.) and figure out other viral pathogens associated with calf diarrhea using metagenomic approach in Korea. In total, 810 feces from KIC were collected,

composed of 526 normal feces (comprising 267 feces with a fecal score of 0 and 259 feces with a fecal score of 1) and 284 diarrheic feces (comprising 178 feces with a fecal score of 2 and 106 feces with a fecal score of 3). All 7 pathogens were detected by PCR in feces and their detection rates and mean fecal scores for each were as follows: BRV (14.0%, 1.41), BCV (3.2%, 1.42), BVDV1 (2.1%, 1.35), BVDV2 (4.9%, 1.33), *C. parvum* (9.8%, 1.66), and *Eimeria* spp. (1.9%, 1.73), and *Giardia* spp. (0.9%, 0.71). Among 7 pathogens, BRV ($p < 0.01$), *C. parvum* ($p < 0.001$), and *Eimeria* spp. ($p < 0.05$) increased as fecal scores increased. Among feces, 21 feces that were negative for all pathogens tested in this study were subjected to high-throughput sequencing to identify viral pathogens associated with calf diarrhea. As a result, the nearly full genomic sequences of bovine kobuvirus, bovine boosepivirus B, bovine astrovirus, bovine parechovirus, bovine torovirus, *C. parvum* virus 1, bovine enterovirus, bovine nebovirus, bovine norovirus and hunnivirus were obtained. This study was the first investigation to identify the presence of BooV, CSpV1, and hunnivirus in KIC and to provide a comprehensive description of the nearly complete genomes of ten novel viruses associated with calf diarrhea in the ROK. The findings of this study would contribute to a better understanding of the epidemiology and molecular characteristics of calf diarrhea-associated pathogens in the ROK.

In this research, clinicopathological changes and the infective pathogens in KIC related with diarrhea were examined. The study integrated insights from both clinical and preventive veterinary medicine related to calf diarrhea, with an

expectation to enrich our understanding of the disease and inform the creation of more effective prevention and treatment strategies. Further research will focus on a broader spectrum of viruses and pathogens, the interplay of co-infections, and innovative prevention methods, ultimately contributing to the improved health and productivity of KIC.

Keywords: Korean indigenous calves, calf diarrhea, hematology, serum biochemistry, pathogens, prevalence, novel viruses

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Contents

List of Figures

List of Tables

List of Abbreviations

APPs	Acute phase proteins
APR	Acute phase response
ALB	Albumin
BA	Basophil
BAstV	Bovine astrovirus
BCV	Bovine coronavirus
BEV	Bovine enterovirus
BNeV	Bovine nebovirus
BNoV	Bovine norovirus
BooV	Boosepivirus
BParV	Bovine parechovirus
BRV	Bovine rotavirus
BToV	Bovine torovirus
BUN	Blood urea nitrogen
BVDV	Bovine viral diarrhea virus
Ca	Calcium
CBC	Complete blood counts
CFTR	Cystic fibrosis transmembrane conductance regulator
cGKII,	cGMP-dependent protein kinase II
cGMP	Cyclic guanylyl monophosphate

Cl	Chloride
CP	Cytopathic
CPE	Cytopathic effect
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EO	Eosinophil
ETEC	Enterotoxigenic *E. coli*
Fib	Fibrinogen
FPT	Failure of passive transfer
GCC,	Guanylyl cyclase-C
GLU	Glucose
GM	Geometric mean
GTP	Guanylyl triphosphate
Hb	Hemoglobin
Hct	Hematocrit
HI	Hemagglutination inhibition tests
Hp	Haptoglobin
HRT-18G	Human rectal tumor cell line
IFT	Immunofluorescence test
Ig	Immunoglobulin
IHC	Immunohistochemistry staining

IPT	Immunoperoxidase test
K	Potassium
KIC	Korean indigenous calves
LAT	Latex agglutination test
LYM	Lymphocyte
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MO	Monocyte
Na	Sodium
Nab	Neutralizing antibodies
NCP	Non-cytopathic
NE	Neutrophil
NGS	Next Generation Sequencing
NSP	Non-structural protein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI	Persistent-infections
PLT	Platelet
RBC	Red blood cell

RETIC	Reticulocyte
ROK	Republic of Korea
RT	Reverse transcriptase
SAA	Serum amyloid A
SD	Standard Deviation
SEM	Standard errors of the mean
SGLT1	Sodium-glucose cotransporters
SP	Serum protein
spp.	Species
SST	Serum seperating tube
Sta	Heat-stable enterotoxin a
USA	The United States of America
VN	Virus neutralization
VP	Viral protein
WBC	White blood cells

General Introduction

The considerable economic impact of calf diarrhea in Korea constitutes a significant challenge for the livestock industry. Despite the pressing need for comprehensive research addressing this critical issue, the existing body of literature is markedly insufficient, highlighting the need for a more in-depth investigation into the multifaceted complexities surrounding calf diarrhea and its management.

To address this research gap, this study embarked on an extensive investigation, the primary objective of which was to mitigate the prevalence of calf diarrhea and, in turn, enhance animal health, welfare, and productivity within the field of Korean livestock, especially in Korean indigenous cattle. This study was divided into several components, focusing on various aspects of calf diarrhea, ranging from blood diagnostic advancements to epidemiological investigations and in-depth diagnosis of pathogens using NGS.

The first study was focused on the development of advanced diagnostic techniques, specifically emphasizing blood analysis for diagnosing calf diarrhea in Korean indigenous calves. By utilizing cutting-edge technologies, state-of-the-art laboratory equipment, and incorporating multidisciplinary approaches, the study aimed to identify reliable biomarkers in blood samples that could serve as sensitive indicators of calf diarrhea. The research involved collecting blood samples from both healthy and diarrheic Korean indigenous calves (KIC), as well as establishing control groups to ensure the accuracy and reliability of these biomarkers.

The second study was focused on an epidemiological study of the

pathogens responsible for causing calf diarrhea in Korean indigenous calves. The primary objective of this investigation was to gain a comprehensive understanding of the prevalence, distribution, and transmission dynamics of these pathogens, which included BRV, BCV, BVDV, *C. parvum*, *E. coli*, *Salmonella* spp., and *Eimeria* spp. Extensive field surveys and collected samples from KIC across various regions and farm types in Korea were performed. These samples were subjected to rigorous laboratory testing to accurately identify the presence of the seven pathogens. Through the systematic analysis of the collected data, the second study could determine the prevalence of each pathogen, identify potential risk factors, and uncover patterns of co-infection among the study population to provide valuable insights into the complex interactions between different pathogens and their potential synergistic effects on disease severity and transmission.

The third study was focused on identifying the etiological agents responsible for diarrhea in KIC, specifically targeting viral pathogens that had been overlooked due to their lack of recognition as major causative agents. To achieve this, NGS technology was employed, which facilitated a comprehensive and unbiased assessment of the entire spectrum of pathogens potentially associated with calf diarrhea in KIC. By emphasizing the discovery of overlooked pathogens using NGS, the third study highlighted the importance of adopting advanced diagnostic methods and embracing a more comprehensive approach to disease investigation. This not only contributed to a deeper understanding of the multifaceted etiology of calf diarrhea in KIC but also underscored the potential of NGS technology in

uncovering previously unrecognized pathogens that may have significant implications for animal health and disease management.

By identifying useful blood biomarkers in calf diarrhea, enhancing the understanding of the multifaceted etiology of calf diarrhea, and uncovering previously unrecognized pathogens associated with calf diarrhea, this research aimed to accumulate and broaden knowledge of calf diarrhea in KIC for the formulation of more precise and efficacious strategies for calf diarrhea prevention, diagnosis, and treatment, ultimately promoting progress in the field of animal health and disease management in KIC.

Literature Review

1.1. The Definition and Mechanism of Calf Diarrhea

Calf scour, or calf diarrhea, is a multifactorial disease that lose their body fluid through the intestine by losing their ability to absorb the fluids from the intestines (malabsorptive) or body fluids passing from the scouring calves' body into the intestines (secretory) (Foster and Smith, 2009). In diarrhea, calves lose their body fluids, electrolytes and other substances from the body, which leads to electrolyte imbalance. This, in turn, causes the concentration of ions and other substances remaining in the fluid to rise, which increases the osmotic pressure of the extracellular fluid. This mechanism results in electrolyte imbalances (e.g., hyponatremia, hyperkalemia, and hypochloremia) and metabolic changes (e.g., decrease in glucose concentration, increase in urea and creatinine concentrations in blood, loss of carbohydrates, and the accumulation of organic acids) (Dratwa-Chałupnik *et al.*, 2012). As dehydration progresses, tissues tend to shrink, skin becomes dry and wrinkled, eyes become shrunken and soft, and the kidneys reduce urine output to conserve water, resulting in waste product accumulation in the blood. Furthermore, impaired kidney productivity leads to changes in plasma ion concentrations and a reduction in plasma pH, which causes of acidosis. Acidosis and dehydration impair the ability to maintain an appropriate body temperature and affect the physical and mental status of the animal. In addition to this, when the plasma pH

is lowered until the cell membrane is depolarized, potassium leaves the cell and increases its concentration in the extracellular fluid (Dratwa-Chałupnik *et al.*, 2012). Membrane potential, which is lowered by this process, affects muscle contraction, making the heart rate irregular, lowers blood pressure, and reduces blood flow into the lungs. This process causes the calf to undergo irreversible shock, eventually leading to a comatose state, and finally death caused by heart failure (Dratwa-Chałupnik *et al.*, 2012).

1.2. Economic Impacts of Calf Diarrhea

Diarrhea can have significant negative effects on the economy of farms. These effects are caused by the various actions to treat the diarrhea, decreased productivity caused by diarrhea, and calf death. In previous studies from the United States of America (USA), the cost of the conventional treatment of calf diarrhea was $11.7 to $12.4 per calf, depending on the usage of antibiotics in feeding milk (Berge *et al.*, 2009). This cost was calculated to include the treatment using antibiotics and electrolyte oral rehydration, as well as the cost of labor ($10/h).

In addition to the cost associated with its treatment, diarrhea can cause negative effects on production in areas such as weight gain and reproduction. Donovan *et al.* (1998) reported that heifers treated for diarrhea, between birth and 6 months of age, had a significantly reduced ($p < 0.01$) daily weight gain of 13.4 g per treatment within this time. Furthermore, in a different study, the 1.1 kg of body

weight gain measured at 3 months was significantly lower in calves with diarrhea than in healthy calves (Windeyer *et al.*, 2014). In addition to the short-term effects (such as those observed over 6 months), a long-term effect of calves suffered from diarrhea before 3 months of age was that they were significantly less likely to calving before 900 days than calves not treated for diarrhea (Waltner-Toews *et al.*, 1986).

Rather than analyzing the specific causes of economic loss due to calf diarrhea, some studies have calculated the approximate amount of damage caused by this disease. According to a previous report, in England the cost of an outbreak of calf diarrhea could be an average of £33 per at-risk calf and costs can be five times that figure when calves die as a result of diarrhea (Vickers and Wright, 2013). Similarly, calf diarrhea occurring in 18% of the population could incur 85 DKK (Danish krone) in the average herd and 260 DKK in a herd of poor reproduction performance (Lorenzen, 2014).

In the ROK, the economic impacts of calf diarrhea on cattle industries were not well analyzed compared to other countries. There was a report in the ROK that the economic damage caused by calf diarrhea would be around 28 million won in the Korean indigenous cattle farm that grew 100 cattle, assuming that the incidence of calf diarrhea is 10% and the mortality rate is 20% (Song *et al*., 2006).

1.3. Risk Factors of Calf Diarrhea

As calf diarrhea is considered one of the most important problems in neonatal calf rearing, there have been many studies on its causes (Klein-Jöbstl *et al.*,

2014; Cho *et al.*, 2013). Calf diarrhea is a multifactorial disease, and most of its causes can be placed into two categories: non-infectious and infectious factors.

1.3.1. Non-Infectious Factors

The classification of non-infectious factors can be further categorized into environmental factors, management factors, and host factors. The environmental factors are related to the housing of calves, including individual calf housing postpartum; the duration of individual calf housing postpartum; the type of individual calf housing; bedding materials; location of calf housing; the frequency and the time at which cleaning is performed; and management after the pre-weaning period. For example, in previous reports, the outdoor housing system showed a significantly higher occurrence of calf diarrhea than a combination of indoor and outdoor housing (Klein-Jöbstl *et al.*, 2014). The management factors included the number of raised cows, the type of feeding, cow-calf separation, the timing of the first colostrum feeding, the quality of the colostrum, dam vaccination, and umbilical care after parturition. Among them, the presence of calving pens or boxes and the cleaning of calving areas after each use were significant factors in reducing calf diarrhea (Klein-Jöbstl *et al.*, 2014). Host factors include the sex of the calves, failure of passive transfer (FPT), and the history of other diseases that they are suffered. The calf should be taking immune factors to overcome diseases, especially immunoglobulin (Ig), from the maternal cattle, which is delivered to the calf through

colostrum. However, because of several reasons, including low colostrum intake or weak calf syndrome, there are cases where IgG less than 10 mg/mL is identified in the calf's serum. These incidences are defined as FPT. Calves with FPT are known to have high mortality rates, and decreased health and longevity (Raboisson *et al.*, 2016). Among various factors, some of non-infectious factors that reported the significant association with calf diarrhea in previous reports were listed in Table 1.

There have been some reports regarding calf diarrhea and non-infectious factors in the ROK. The periodical changes of bedding materials, calving on September and October, the removal of diarrheic feces in calving pens, and clean and well-ventilated cow house with dry bedding showed reduced the incidence of calf diarrhea (Lee *et al*., 2007). There are other reports that cleaning frequency, vaccination, and individual calf space showed good effects on reducing calf diarrhea (Cho *et al*., 2017).

Table 1. Non-infectious factors of calf diarrhea in previous reports

Categories	Variables	References
Environmental factors	Type of individual housing	Bentali *et al*., 1999; Gulliksen *et al*., 2009; Klein-Jöbstl *et al*., 2014
	Bedding materials	Al Mawly *et al*., 2015; Panivivat *et al*., 2004
	Frequency of cleaning	Bentali *et al*., 1999; Klein-Jöbstl *et al*., 2014
	Cleanliness	Bentali *et al*., 1999;
	Other animals presence	Klein-Jöbstl *et al*., 2014
	Placement of individual housing	Bentali *et al*., 1999; Klein-Jöbstl *et al*., 2014
Management factors	The number of cows (size of farms)	Gulliksen *et al*., 2009; Klein-Jöbstl *et al*., 2014; Lievaart *et al*., 2013
	The quality of colostrum	Abuelo *et al*., 2019; Al Mawly *et al*., 2015
	Dam vaccination	Al Mawly *et al*., 2015; Bentali *et al*., 1999
	Management people	Al Mawly *et al*., 2015
	Recent introduction of animals to the farm	Bentali *et al*., 1999; Gulliksen *et al*., 2009
Host factors	Failure of transfer of passive immunity	Lievaart *et al*., 2013; Raboisson *et al*., 2016
	Diseases history in farms	Al Mawly *et al*., 2015; Klein-Jöbstl *et al*., 2014
	Suckling reflex	Bentali *et al*., 1999

1.3.2. Infectious pathogens

Infectious pathogens have been known as major factors in calf diarrhea (Cho *et al.*, 2013; Izzo *et al.*, 2011). More than 14 pathogens are known to cause calf diarrhea so far; however, the main pathogens are considered to be bovine rotavirus group A (BRV), bovine coronavirus (BCV), bovine viral diarrhea virus (BVDV), *E. coli*, *Salmonella* species (spp.), *Cryptosporidium parvum*, and *Eimeria* spp. (Cho and Yoon, 2014; Foster and Smith, 2009).

1.3.2.1. Bacteria

1.3.2.1.1. *Escherichia coli*

Escherichia coli has been described as a significant infectious agent of neonatal calf diarrhea, resulting in high morbidity and mortality rates. It is also responsible for a significant amount of money and time spent on diarrhea treatments, as well as poor growth performance. *Escherichia coli* are classified according to their virulence type; namely: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli*, and shigatoxigenic *E. coli*, which include a subgroup comprising enterohemorrhagic *E. coli*, enteroinvasive *E. coli*, enteroaggregative *E. coli*, and enteroadherent *E. coli* (Nagy and Fekete, 2005).

Among them, ETEC, which produces K99 adhesion antigens and heat stable toxins, is the most common cause of neonatal calf diarrhea, especially in 4-day-old calves (Nataro and Kaper, 1998). Calf diarrhea due to ETEC infection is observed

mainly within the first 4 days of the calf's life. This is because the pH of the abomasum in newborn calves is approximately 6-7, enough for ingested ETEC to survive and pass through to the small intestine. If ETEC existed in the environment around the farms and was ingested by a calf, the ETEC would colonize the gut with normal flora, and then move to the caudal part of the gut. K99, the factor responsible for the attachment and colonization of ETEC in the gut, is expressed at pH levels below 6.5. ETEC usually begin to colonize the distal small intestine first, specifically the ileum. After colonization, they produce heat-stable toxins, which can stimulate the secretion of Cl by activating the cystic fibrosis transmembrane conductance regulator (Foster and Smith, 2009). Sodium and bicarbonate are also secreted due to the induction of a tyrosine kinase (Sellers *et al.*, 2005) (Fig. 1).

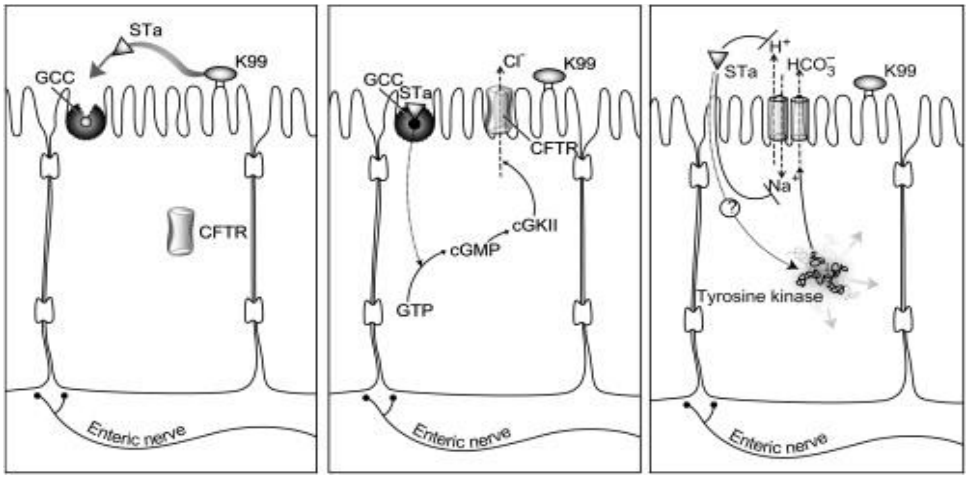


Fig. 1. Schematic representation of secretory diarrhea by heat stable toxin (STa) produced by K99 Enterotoxigenic *Escherichia coli* (Adapted from Foster and Smith, 2009). CFTR, cystic fibrosis transmembrane conductance regulator; cGKII, cGMP-dependent protein kinase II; GCC, guanylyl cyclase-C; Sta, heat stabile toxin; cGMP, cyclic guanylyl monophosphate; GTP, guanylyl triphosphate.

1.3.2.1.2. *Salmonella* species

Various *Salmonella* spp. have been known to affect the health of calves. Among *Salmonella* spp., *S. enterica* serovar *Typhimurium* (*S. Typhimurium*) and serovar *Dublin* (*S. Dublin*) are the most common etiologic agents that cause salmonellosis in cattle (Hughes *et al.*, 1971). The mechanism of *Salmonella* virulence has been well studied in previous reports. According to these reports, after *Salmonella* infects the calf's body via the fecal-oral route or the mucosa of the upper respiratory tract and conjunctiva, the organism invades intestinal epithelial cells through M cells, and colonizes enterocytes and tonsillar tissues. After this, it spreads throughout the whole body through mononuclear cells and phagocytes (Holt, 2000; Tsolis *et al.*, 1999).

After a *Salmonella* infection, depressed mentation and loss of appetite would be the first clinical signs, followed by high fever and diarrhea 2-3 days post-infection. *Salmonella* infection in neonatal calves usually occurs between 10-90 days of age but can also be seen in older calves and adult cattle (Fossler *et al.*, 2005). The organism can be shed by the infected host over various periods and times, depending on the calf's age, stress, passive immunity from maternal cattle, challenge dose, and the severity of the infection and clinical signs (Mohler *et al.*, 2009).

1.3.2.2. Viruses

1.3.2.2.1. Bovine Rotavirus

Bovine rotavirus is one of the first identified viral causes of diarrhea and BRV group A is the major causes in calf diarrhea among 10 groups (A to J) (Foster and Smith, 2009; Park *et al*., 2022). Bovine rotavirus genomes consist of 11 segments of double-stranded RNA, surrounded by a triple-layered virus particle, encoding six structure viral proteins (VP1, VP2, VP3, VP4, VP6, and VP7) and 5-6 non-structured proteins (NSP1, NSP2, NSP3, NSP4, NSP5, and NSP6). Among the structural particles of the virus, VP4 and VP7 proteins, which form the outer layer of BRV, are important proteins that produce the neutralizing antibodies associated with BRV. These proteins are also used in virus classification systems; namely, P types (protease sensitive, VP4) and G types (glycoprotein, VP7) (Matthijnssens *et al.*, 2011).

Bovine rotavirus-induced calf diarrhea occurs when the calf is exposed to the virus via contaminated milk, water, or feed materials (Dhama *et al.*, 2009). After the virus is ingested by the calves, the outer capsid of the virus would be cleaved by intestinal proteases, such as chymotrypsin, in order to increase the chances of a successful infection (Ramig, 2004). Ingested viruses have an incubation period of 12-24 hours and destroy mature enterocytes. This is followed by intestinal surface loss, causing nutrients, including glucose and carbohydrates, to remain in the lumen. This, in turn, results in osmotic high pressure in the fluid, leading to malabsorptive

diarrhea. In addition, BRV can secrete the viral toxin, NSP4, which is known to alter intracellular calcium (Ca) concentration, resulting in the translocation of disaccharidases. This process slows the digestion of carbohydrates, promote chloride (Cl) secretion, and directly inhibit sodium-glucose cotransporters (SGLT1), which have functions in water absorption (Fig. 2) (Chauhan *et al.*, 2008). Bovine rotavirus-induced diarrhea usually occurs in calves less than 3 weeks of age.

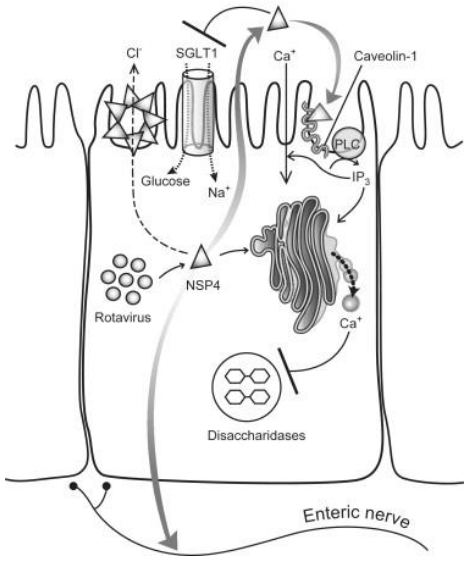


Fig. 2. Schematic representation of secretory diarrhea by bovine rotavirus (Adapted from Foster and Smith, 2009). SGLT, sodium-glucose transport proteins; NSP4, non-structural proteins 4.

1.3.2.2.2. Bovine Coronavirus

Bovine coronavirus is one of the most prevalent viruses in cattle. It can cause negative health effects for calves and cattle, as well as economic losses in the cattle industry. Bovine coronavirus-associated syndrome can be divided into three groups:

BCV-associated respiratory diseases, BCV-associated calf diarrhea, and BCV-associated winter dysentery in cattle (Boileau and Kapil, 2010). Notably, BCV-associated diarrhea has also been found in adult cattle during warm seasons in the ROK (Park *et al.*, 2006). There has been debate about biological, antigenic, and genetic characteristics of these viruses; however, their relationship remains unclear because of their size and their ability to evolve (Boileau and Kapil, 2010). In this paper, only BCV-associated calf diarrhea is discussed.

The pathophysiology of BCV-associated calf diarrhea is similar to that of BRV in calves. After the virus is ingested through the fecal-oral route, BCV infects the small intestine and colon by attaching to enterocytes using spike protein and hemagglutinin glycoproteins. It then destroys intestinal villi, crypt enterocytes, and colonocytes, resulting in secretory and malabsorptive diarrhea (Foster and Smith, 2009). Diarrhea begins with virus entry into the cell, usually 2 days after ingestion, and continues for 3 to 6 days (Clark, 1993). As BCV can infect not only the small intestine but also the large intestine, diarrhea caused by BCV would be more severe than diarrhea caused by BRV.

1.3.2.2.3. Bovine Viral Diarrhea Virus

Bovine viral diarrhea virus is classified as *Flaviviridae*, which consists of viruses with an inner capsid or shell containing the viral genome, surrounded by a pleomorphic outer lipid envelope (Becher *et al.*, 1999). Bovine viral diarrhea virus

can be segmented into three distinct species, *Pestivirus* A (BVDV-1) which contains at least 22 subgenotypes (1a-1v), *Pestivirus* B (BVDV-2), and *Pestivirus* H (also known as BVDV-3 or Hobi-like Pestiviruses), with the latter two further subdivided into four subtypes each, distinctions made based on the genetic sequence in the 5' untranslated region (UTR) (Yang *et al*., 2022). They may also exist in two biotypes: cytopathic (CP) and non-cytopathic (NCP). CP BVDV, as the name suggests, shows cytopathic effects (CPE) in cultured epithelial cells, and is related to mucosal disease in calves, a highly fatal form of BVDV infection. Meanwhile, NCP BVDV does not show CPE in cultured epithelial cells and is commonly found in nature (Ridpath, 2010). Mucosal disease in calves caused by CP BVDV is believed to be due to the genetic mutation of NCP BVDV to CP BVDV brought on by massive recombination between the viral and host genomes in the NS2-NS3-NS4 genome region (Colitti *et al.*, 2019).

Diarrhea, leukopenia, pyrexia, depression, anorexia, gastrointestinal erosions, and hemorrhages were observed in calves with BVDV infection. Additionally, respiratory and reproductive diseases have also been reported in previous reports worldwide (Goyal and Ridpath, 2008). In particular, the clinical features of this disease differ depending on the stage of gestation when the fetus is infected. While abortion and weak calves occur in late gestation, mummification, abortion, congenital malformations, or persistent infections (PI) in animals have been observed within 42-125 days of gestation.

1.3.2.3. Protozoa

1.3.2.3.1. *Cryptosporidium parvum*

Cryptosporidium parvum is an important protozoan parasite that causes calf diarrhea. *C. parvum* can be transmitted to the host through direct contact with infected hosts, or indirectly via environmental contamination or ingestion of contaminated food or water (Thomson *et al.*, 2017). After ingestion of the oocyst of *C. parvum* (Fig. 3A), oocyst excystation of sporozoites is triggered by exposure to low pH in the gastrointestinal tract, body temperature, and bile salts (Fig. 3B). The sporozoites attach to the intestinal epithelial cells, especially in the ileocecal junction, and then transform into tropozoites (Fig. 3C). Afterwards, the organisms start asexual reproduction (Fig. 3D) and become Type I meronts (Fig. 3E). The type I meronts then release merozoites, which can become either Type I or Type II meronts (Fig. 3F). Furthermore, the Type II meront can eventually become a microgamont (Fig. 3G) or a macrogamont (Fig. 3H). After this process they start sexual reproduction, which cause the zygote (Fig. 3I) to differentiate into thick-walled oocysts (Fig. 3J) and thin-walled oocysts. The thick-walled oocysts can then be passed out with feces to contaminate the environment, while the thin-walled oocysts (Fig. 3K) mainly act as agents involved in autoinfection.

An infection of *C. parvum* is known to cause diarrhea through two mechanisms. After *C. parvum* infection, the loss of epithelial cells and villous atrophy occurs. This process results in impaired absorption of Na, Cl, and other

substances, described as malabsorptive diarrhea (Heine *et al.*, 1984). The secretion of prostaglandin E_2 (PGE_2) in epithelial enterocytes, and the secretion of PGE_2 and PGI_2 in mesenchymal cells occur resulting in increased levels of intracellular calcium and cyclic adenosine monophosphate. This is then followed by the activation of anion secretion, and the inhibition of Na and Cl absorption (Argenzio *et al.*, 1993).

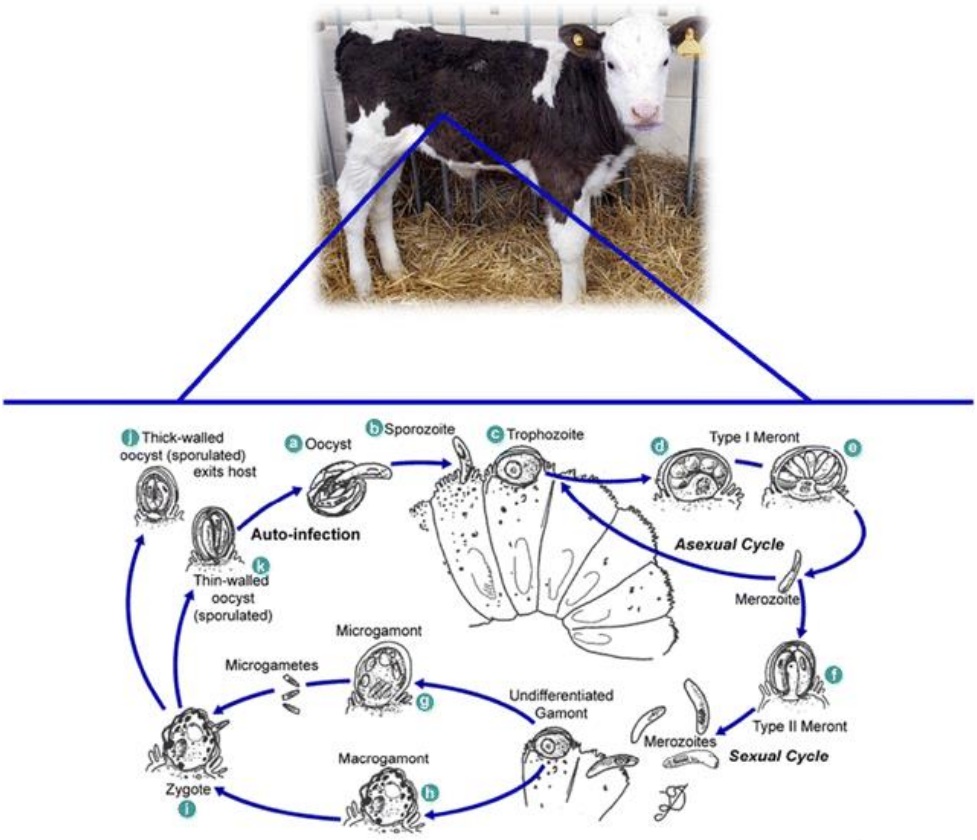


Fig. 3. Life cycle of *Cryptosporidium* (Adapted from Thomson *et al*., 2017).

1.3.2.3.2. *Eimeria* species

Eimeria spp. are also one of the most important protozoan parasites that cause calf diarrhea. Infection with *Eimeria* spp. in calves is also called calf coccidiosis and, while it is known to infect cattle of all ages, it has a major impact on calves less than 1 year of age (Gillhuber *et al.*, 2014). Various *Eimeria* species have been reported; however, *E. zuernii*, *E. bovis*, and *E. alabamensis* are pathogenic to calves (Keeton and Navarre, 2018). The life cycle of *Eimeria* spp. in calves is described (Daugschies and Najdrowski, 2005) as follows: After a calf ingests sporulated oocytes, the sporozoites are released and infect intestinal cells to become merozoites. Merozoites can then infect other intestinal cells and either multiply or transform into microgametocytes or macrogametocytes. Microgametocytes fertilize macrogametocytes, which become unsporulated oocysts during the sexual phase. Oocysts rupture host cells after maturation and are subsequently released into the lumen of the intestine before being shed in feces.

The severity of the clinical signs by *Eimeria* spp. depends on the number of oocysts that calves ingest, as well as the species of *Eimeria*, the age of the host, the environmental temperature, the humidity, and the immunity of the calves (Jolley and Bardsley, 2006). Typical signs of calves with pathogenic *Eimeria* spp. are diarrhea, anorexia, depression, weakness, abdominal pain, dehydration, pale mucous membranes, acute weight loss, as well as straining to defecate and rectal prolapse (Keeton and Navarre, 2018).

1.3.3. Prevalence of Infectious Pathogens of Calf Diarrhea

1.3.3.1. The Republic of Korea

The prevalence reports of various infectious pathogens causing calf diarrhea in the ROK are described (Tables 2~4). Various bacteria (*E. coli* K99, *Salmonella* spp., *Clostridium perfringens* type A) in calf diarrhea were reported (Table 2). The infection rates of *E. coli* K99, *Salmonella* spp., and *Clostridium perfringens* type A were 0.0~0.6% (Chon *et al*., 2007; Koh *et al*., 2019; Ryu *et al*., 2020a), 0.0~0.6% (Koh *et al*., 2019; Lee el al., 2020) and 8.0~37.5% (Koh *et al*., 2019; Chon *et al*., 2007), respectively.

Various viruses (BRV, bovine norovirus (BNoV), BCV, and bovine torovirus (BToV)) in calf diarrhea were also reported (Table 3). The infection rates of BRV, BNoV, BCV, and BToV were reported to be 1.9~43.0% (Koh *et al*., 2019; Lee *et al*., 2020; Ryu *et al*., 2019), 1.8~9.3% (Koh *et al*., 2019; Lee *et al*., 2019; Park *et al*., 2007; Ryu *et al*., 2020b), 3.0~15.6% (Koh *et al*., 2019; Lee *et al*., 2019; Park *et al*., 2008; Park *et al*., 2007; Ryu *et al*., 2020b; Yook *et al*., 2009), and 1.8~8.0% (Koh *et al*., 2019; Lee *et al*., 2019; Park *et al*., 2008; Ryu *et al*., 2020b), respectively.

Various protozoan pathogens (*C. parvum*, *Eimeria* spp., and *Giardia* spp.) were reported (Table 4). The infection rates of *C. parvum*, *Eimeria* spp., and *Giardia* spp. were reported to be 0.8~10.0% (Chon *et al*., 2007; Kim *et al*., 2018; Koh *et al*., 2019; Lee *et al*., 2019), 12.2~74.6% (Koh *et al*., 2019; Lee *et al*., 2018; Lee *et al*., 2019; Lee *et al*., 2020), 4.9~13.0% (Koh *et al*., 2019; Lee *et al*., 2018; Lee *et al*.,

2019; Lee *et al*., 2020).

1.3.3.2. Other Countries

The prevalence reports of various infectious pathogens causing calf diarrhea in other countries are described (Tables 2~4). Various bacteria (*E. coli* K99, *Salmonella* spp., *C. perfringens* type A, *Campylobacter jejuni*) in calf diarrhea were reported (Table 2). The infection rates of *E. coli* K99, *Salmonella* spp., *C. perfringens* type A and *C. jejuni* were 0.7~13.1% in other countries including Netherlands, USA, and Turkey (Bartels *et al*., 2010; Cho *et al*., 2013; Ok *et al*., 2009; Sham *et al*., 2012 Umpiérrez *et al* 2016), 4.1~46.7% in other countries including Bangladesh, Iraq, USA and Egypt (Abdullah *et al*., 2013; Anwarullah *et al*., 2014; Cho *et al*., 2013; El-Seedy *et al*., 2016), 0.0~54.0% in other countries including India, Netherlands and USA (Athira *et al*., 2018; Bartels *et al*., 2010; Cho *et al*, 2013), and 11.0~14.4% in other countries including Mozambique and Austria (Acha *et al*., 2004; Klein-Jöbstl *et al*., 2016), respectively.

Various viruses (BRV, BNoV, BCV, BToV, and BNeV) in calf diarrhea were also reported (Table 3). The infection rates of BRV, BNoV, BCV, BToV and BNeV were reported to be 12.2~27.0% in other countries including Algeria, Netherlands, and Iran (Ammar *et al*., 2014; Bartels *et al*., 2010; Mayameei *et al*., 2010), 4.0~39.5% in other countries including USA, Iran, Egypt and Turkey (Cho *et al*., 2013; Pourasgari *et al*., 2018; Mohamed *et al*., 2017; Turan *et al*., 2018), 3.1~24.0% in other countries including Algeria, USA, Iran, and Brazil (Ammar *et al*., 2014; Cho

et al., 2013; Lotfollahzadeh *et al*., 2020; Pourasgari *et al*., 2018; Mayameei *et al*., 2010; Takiuchi *et al*., 2006), 1.1~6.3% in other countries including Turkey, USA, China and Brazil (Gülaçt *et al*., 2014; Cho *et al*., 2013; Shi *et al*., 2020; Nogueira *et al*., 2013), and 20.0~ 41.8% in other countries including France, USA and China (Kaplon et al., 2011; Cho et al., 2013; Guo et al., 2019), respectively.

Various protozoan pathogens (*C. parvum*, *Eimeria* spp., and *Giardia* spp.) were also reported in other countries (Table 4). The infection rates of *C. parvum*, *Eimeria* spp., and *Giardia* spp. were reported to be 15.1~69.6% in other countries including Netherlands, USA, Ethiopia, India and Canada (Bartels *et al*., 2010; Cho *et al*., 2013; Hailu *et al*., 2020; Singh *et al*., 2006; Trotz-Williams *et al*., 2005), 12.0~83.7% in other countries including Germany, Brazil, India, Austria, Kenya and Ethiopia (Bangoura *et al*., 2011; Bruhn *et al*., 2012; Das *et al*., 2015; Koutny *et al*., 2012; Peter *et al*., 2016; Tamrat *et al*., 2020), and 13.3~74.2% in other countries including China, Ethiopia, Egypt, Vietnam and Algeria (Feng *et al*., 2019; Hailu *et al*., 2020; Naguib *et al*., 2018; Nguyen *et al*., 2016; Ouchene *et al*., 2014), respectively.

1.3.3.3. The Comparison of Infectious Pathogens of Calf Diarrhea Between the Republic of Korea and Worldwide

While a variety of bacteria have been identified both at a national and global scale, the infection prevalence of *Salmonella* spp., in particular, demonstrates a stark

contrast. Specifically, the infection prevalence in domestic cases falls within the range of 0.0-0.6%, a figure considerably lower than those reported internationally, which range from 4.1~46.7%. Furthermore, instances of *C. jejuni* detection in cases of calf diarrhea have not yet been documented in Korea. In terms of viral prevalence, there is no marked discrepancy between national and international rates. However, the global reports exhibit a broader spectrum of viruses, including *Hunnivirus* and *Boosepivirus* (Nagai et al., 2015a; Wang et al., 2022).

Table 2. The prevalence of bacterial pathogens regarding calf diarrhea in the Republic of Korea and worldwide

Microbe	Pathogens	The Republic of Korea		Worldwide	
		Infection rates (%)	References	Infection rates (%)	References
Bacteria	*E. coli* K99	0.0~0.6	Chon *et al*., 2007; Koh *et al*., 2019; Ryu *et al*., 2020a	0.7~13.1	Bartels *et al*., 2010; Cho *et al*., 2013; Ok *et al*., 2009; Sham *et al*., 2012 Umpiérrez *et al* 2016
	Salmonella spp.	0.0~0.6	Koh *et al*., 2019; Lee el al., 2020	4.1~46.7	Abdullah *et al*., 2013; Anwarullah *et al*., 2014; Cho *et al*., 2013; El-Seedy *et al*., 2016
	C. perfringens type A	8.0~37.5	Koh *et al*., 2019; Chon *et al*., 2007	0.0~54.0	Athira *et al*., 2018; Bartels *et al*., 2010; Cho *et al*, 2013
	C. jejuni	Not reported		11.0~14.4	Acha *et al*., 2004; Klein-Jöbstl *et al*., 2016; Giaconobi *et al*., 1993

C. perfringens type A, *Clostridium perfringens* type A; *C. Jejuni*, *Campylobacter jejuni*; *E. coli, Escherichia coli*.

Table 3. The prevalence of viral pathogens regarding calf diarrhea in the Republic of Korea and worldwide

Microbe	Pathogens	The Republic of Korea		Worldwide	
		Infection rates (%)	References	Infection rates (%)	References
Virus	Bovine rotavirus	1.9~43.0	Koh *et al*., 2019; Lee *et al*., 2020; Ryu *et al*., 2019b	12.2~27.0	Ammar *et al*., 2014; Bartels *et al*., 2010; Mayameei *et al*., 2010
	Bovine norovirus	1.8~9.3	Koh *et al*., 2019; Lee *et al*., 2019; Park *et al*., 2007; Ryu *et al*., 2020b	4.0~39.5	Cho *et al*., 2013; Pourasgari *et al*., 2018; Mohamed *et al*., 2017; Turan *et al*., 2018
	Bovine coronavirus	3.0~15.6	Koh *et al*., 2019; Lee *et al*., 2019; Park *et al*., 2008; Park *et al*., 2007; Ryu *et al*., 2020b; Yook *et al*., 2009	3.1~24.0	Ammar *et al*., 2014; Cho *et al*., 2013; Lotfollahzadeh *et al*., 2020; Pourasgari *et al*., 2018; Mayameei *et al*., 2010; Takiuchi *et al*., 2006
	Bovine torovirus	1.8~8.0	Koh *et al*., 2019; Lee *et al*., 2019; Park *et al*., 2008; Ryu *et al*., 2020b	1.1~6.3	Cho *et al*., 2013; Gülaçtı*et al*., 2014; Shi *et al*., 2020; Nogueira *et al*., 2013
	Boosepivirus	Not reported		6.0 -23.0	Hause *et al*., 2021 Wang *et al*., 2022 Nagai *et al*., 2015a

Table 4. The prevalence of protozoan pathogens regarding calf diarrhea in the Republic of Korea and worldwide

Microbe	Pathogens	The Republic of Korea		Worldwide	
		Infection rates (%)	References	Infection rates (%)	References
Protozoa	*C. parvum*	0.8~10.0	Chon *et al*., 2007; Kim *et al*., 2018; Koh *et al*., 2019; Lee *et al*., 2019	15.1~69.6	Bartels *et al*., 2010; Cho *et al*., 2013; Hailu *et al*., 2020; Singh *et al*., 2006; Trotz-Williams *et al*., 2005
	Eimeria spp.	12.2~74.6	Koh *et al*., 2019; Lee *et al*., 2018; Lee *et al*., 2019; Lee *et al*., 2020	12.0~83.7	Bangoura *et al*., 2011; Bruhn *et al*., 2012; Das *et al*., 2015; Koutny *et al*., 2012; Peter *et al*., 2016; Tamrat *et al*., 2020
	Giardia spp.	4.9~13.0	Koh *et al*., 2019; Lee *et al*., 2018; Lee *et al*., 2019; Lee *et al*., 2020	13.3~74.2	Feng *et al*., 2019; Hailu *et al*., 2020; Naguib *et al*., 2018; Nguyen *et al*., 2016; Ouchene *et al*., 2014

1.4. Diagnosis of Calf Diarrhea

The precise diagnosis of calf diarrhea has a very important impact on the health of the calves with clinical symptoms, as well as the health of the animals being raised together. It is also the basis for the treatment administered by veterinarians. In order to diagnose diarrhea in calves, clinical diagnosis in the field by clinical veterinarians and the diagnosis in the laboratories by diagnosticians should be evaluated together.

1.4.1. Diagnosis Based on Clinical Signs

Diagnosis performed by clinical veterinarians should include interviews with key individuals (owners, caretakers, veterinarians, nutritionists, and other stakeholders). It should also identify factors responsible for the disease outbreak and investigate the health of the affected host (Smith, 2012). Of all the information needed for diagnosis, two of the most important pieces of information for the diagnosis of calf diarrhea are the age of the affected calves and the clinical features associated with their diarrhea. These two factors can be useful in the diagnosis of calf diarrhea, because each pathogen causing calf diarrhea has its preferred host age and typical clinical signs, although not all cases are applicable (Table 5) (Millemann, 2009).

Table 5. Elements for the differential clinical diagnosis of neonatal calf diarrhea (Adapted from Millemann, 2009)

Mean age of affected calves	Clinical signs	Probable etiological diagnosis
1-3 days	Very liquid diarrhea, Yellow, Rapid and important dehydration, Weakness, Cold extremities	Colibacilosis (ETEC = K99 *E. coli*)
4-11 days	Mucoid diarrhea, Hyperthermia, Anorexia, Abdominal pain, Progressive dehydration	Rotavirosis, Coronavirosis, Cryptosporidiosis
>11 days	Very liquid diarrhea with blood traces, Severe hyperthermia	Salmonellosis
>18 days	Black diarrhea, +/- blood and colic, Mucoid diarrhea, hyperthermia, Ptyalism, Anorexia, Epiphora	Coccidiosis BVDV

1.4.1.1. Physical Examination

The physical examination of calves with diarrhea includes assessing the dehydration status, fecal consistency, physical status, body temperature, and other symptoms. Dehydration status can be assessed through the determination of skin elasticity and enophthalmos. Skin elasticity is determined by pinching a fold of skin over the cervical region, rotating it 90 degrees and observing the time elapsed before it returns to normal; while enophthalmos is determined by measuring the distance between the globe and palpebral conjunctiva (Dawes *et al.*, 2014). Fecal consistency is the evaluation of the amount of water in feces, which is directly related to calf diarrhea. The fecal consistency scoring system is a 4-level scoring system based on the degree of fluidity of the feces (score 0, normal; score 1, semi-formed; score 2, loose but bedding; score 3, watery). Scores 2 and 3 are considered symptoms of diarrhea (McGuirk, 2008). Moreover, physical status can be evaluated as the level of response to stimuli. The more progression, or the more the dehydration associated with the disease becomes severe, the weaker the tendency (Lee *et al.*, 2020). In addition, as calf diarrhea continues, it is also important to observe normal health status, including nasal discharge, eye discharge, and ear posture, as symptoms may occur due to physiological changes in other organs in addition to the digestive organs.

1.4.1.2. Blood parameters

One of the more important components in the diagnosis of calf diarrhea is

the evaluation of the condition of the affected host. There are many ways to evaluate the condition of calves, including measuring blood variables (e.g., complete blood counts (CBC), serum biochemistry, and acute phase proteins (APPs)) that are used for accurate health status evaluation.

Various changes, including metabolic acidosis, dehydration, and inflammation, are found in calves with diarrhea and these changes can be evaluated using blood variables. Metabolic acidosis in calf diarrhea, caused by the loss of carbohydrates and the accumulation of organic acids, can be detected by low pH values, low blood plasma concentrations, and high D-lactic acid concentrations in blood (Guzelbektes *et al.*, 2007; Schlerka *et al.*, 1996). Water and electrolyte losses can be directly evaluated using the concentrations of sodium, chloride, potassium, and bicarbonate in the blood. Moreover, low blood zinc concentration due to loss of zinc, low resorption, and increased utilization by the immune system and stores in tissues has been reported in calf diarrhea. This is in addition to high blood copper concentration caused by acute phase responses (APR) and antioxidant synthesis (Ranjan *et al.*, 2006). High levels of total serum protein (SP), blood urea nitrogen (BUN), glucose (GLU), hemoglobin (Hb), hematocrit (Hct), and white blood cells (WBC), brought on by dehydration, have also been reported in previous studies (Seifi *et al.*, 2006).

To evaluate the severity of inflammation and levels of metabolic activities observed during calf diarrhea, an effective method is to evaluate the acute phase proteins. APPs are proteins that change during APR caused by infection, tissue injury,

neoplastic growth, or immunological disorders (Tothova *et al.*, 2014). However, because APPs change according to the various diseases, it is difficult to use APPs as a cause or diagnosis of disease because of their low specificity. Nevertheless, it is highly sensitive to infections and inflammatory reactions. Because of these characteristics, APPs have been widely used as auxiliary methods in the diagnosis of diseases (Kent, 1992). Various APPs have been reported in bovine species (Table 6) (Ceciliani *et al.*, 2012), but in particular, haptoglobin (Hp), serum amyloid A (SAA), fibrinogen (Fib), and ceruloplasmin have mainly been studied in relation to diarrhea (Balikci and Al, 2014; Hajimohammadi *et al.*, 2013).

One thing to consider when using blood variables for the diagnosis of calf diarrhea is that most of the blood values change because of various factors such as age, the host species, and measurement methods during the calf period. Therefore, laboratories measuring these blood variables should have their own reference range when using blood variables in the diagnosis of calf diarrhea.

Table 6. Bovine acute phase proteins (Adapted from Ceciliani *et al*., 2012)

Acute phase proteins	Category	Functions/comments
Haptoglobin (Hp)	Major	Binds hemoglobin
Serum amyloid A (SAA)	Major	Opsonin binds cholesterol
Mammary associated serum amyloid A3 (MSAA3)	Major	Milk APP; Opsonin; Mucin stimulant
α1Acid glycoprotein (AGP)	Moderate	Transports molecules in plasma modulates innate and adaptive immunity
Lipopolysaccharide binding protein (LBP)	Moderate	Binds LPS; activates innate immunity
Ceruloplasmin	Moderate/minor	Copper containing; binds iron; oxidase activity
Fibrinogen	Moderate	Forms fibrin of blood clot
Inter α trypsin inhibitor H4 (ITIH4)	Moderate	Protease inhibitor
Fetuin (α2 HS glycoprotein)	Moderate	Bone growth, fetal development
α1anti-proteinase (anti trypsin)	Minor	Protease inhibitor
Albumin	Negative	Binds fatty acid, bilirubin, osmotic pressure
Paraoxonase	Negative	Oxidase inhibitor
Lipoprotein	Negative	lipid transport; assay by cholesterol concentration
Retinol binding protein	Negative	Transport of vitamin A; assay by Vitamin A concentration

1.4.2. Diagnosis Based on Pathological Findings

1.4.2.1. Field Necropsy in Calf Diarrhea

In farms that were severely affected by calf diarrhea, sacrificed, or euthanized calves could provide useful information for further diagnosis. During necropsy, damage done to the organs that might have been caused by calf diarrhea should be observed. For example, the presence of fat stores around the kidney and the size of the thymus can provide information about the length of time of the illness. Meanwhile, a change in the contents, size, color, and thickness of the intestinal tract can provide information about past oral medication, infections, and other reasons to rule out other diseases that should be distinguished from the symptoms caused by diarrhea (Blanchard, 2012). In addition, tissue samples should be collected for laboratory diagnosis (pathogen detection and histopathological examination) during necropsy.

1.4.2.2. Sample Collection for Laboratory Diagnosis in Calf Diarrhea

After evaluation by a clinical veterinarian, laboratory diagnosis should be performed to verify the exact causes. Appropriate sampling is essential in laboratory diagnosis, and each sample must be stored and processed according to the diagnostic method. Samples suitable for the diagnosis of calf diarrhea include feces, blood, intestinal tissues (duodenum, jejunum, ileum, cecum, and colon), and other organs

(liver, lung, heart, kidney, thymus, and spleen). Feces or fecal swabs should be collected directly from diarrheic calves to prevent environmental contamination and blood needs to be collected before treatment. Tissue specimens from euthanized calves or sentinel calves in diarrhea-affected farms are also very useful for the diagnosis of calf diarrhea. All samples were properly stored and transported with refrigeration for precise laboratory diagnoses. The detection methods and specimen type for the diagnosis of calf diarrhea agents are described in Table 7 (Blanchard, 2012).

Table 7. Detection methods and specimen type for the diagnosis of calf diarrhea agents (Adapted from Blanchard, 2012)

Pathogens	Specimens	Test methods
Clostridium perfringens Type C	Small intestine contents (frozen) Feces (if alive)	Culture and genotyping, toxin: agELISA, PCR
Clostridium difficile	Colon contents or feces (fresh or frozen) Intestine lesion (fixed) Colon and ileum (fixed)	Culture and toxin testing HP (tissue)
E. coli, K99 (F5)	Feces and/or ileum content Ileum (fixed)	agELISA Culture IC, LA, SA, PCR HP
E. coli, attaching and effacing	Ileum and colon (fresh and fixed)	HP and culture (typing, PCR eae gene and/or toxin detection)
Salmonella spp.	Feces Intestine Tissues	Culture, IC, PCR
Coronavirus	Colon and ileum (fresh-FA) Feces	FA, HP agELISA, EM, IC, PCR
Rotavirus	Feces small intestine (fresh- FA)	agELISA, EM, FA, IC, LA, PCR
Cryptosporidium spp.	Feces	AF, agELISA, FA, Flotation, IC, PCR
Coccidia	Feces Colon	Flotation McMaster's HP, scraping- direct (unfixed)
Nematodes	Feces Abomasum, intestine	Flotation McMaster's HP, Gross examination

Abbreviations: AF, acid-fast stain on direct smear; agELISA, antigen ELISA; EM, electron microscopy; FA, fluorescent antibody test; HP, histopathology; IC, immune-chromatography assay (lateral-flow agELISA); ICP, inductively coupled plasma atomic emission spectroscopy; LA, latex agglutination; PCR, polymerase chain reaction with nucleic acid probe; SA, slide agglutination.

1.4.3. Diagnosis Based on Identified Pathogens

1.4.3.1. Laboratory Diagnosis of Calf Diarrhea

Laboratory diagnosis requires the identification of the causative agents of calf diarrhea and their findings in the affected lesions. When identifying the causative agents of calf diarrhea, pathogen isolation from the affected host is considered the gold standard. However, it is very difficult to isolate pathogens because numerous experimental equipment, materials, knowledge, and skills are necessary for the properties of each causative agent. Therefore, it is considered acceptable to use alternative methods to identify pathogens directly or indirectly in samples collected from affected hosts, using some experimental procedures (Cho and Yoon, 2014). Among the various methods for the isolation and detection of various pathogens that can cause calf diarrhea, the methods used for the seven pathogens mentioned above are discussed.

1.4.3.2. Laboratory Diagnosis of Bovine Rotavirus

The diagnosis of BRV in the laboratory is mainly focused on the isolation or identification of pathogens from the affected host using various methods. BRV is isolated from the affected host feces or tissue samples taken from the small intestine in the rotavirus-specific cell line, MA-104 (epithelial monkey kidney cells) or TF-104 (a cloned derivative of MA-104 monkey kidney cells) (Table 8). BRV can also be detected directly by electron microscopy (Steele *et al.*, 2004). In addition, the

immunofluorescence test (IFT), immunoperoxidase test (IPT), viral-RNA-based polyacrylamide gel electrophoresis (PAGE), latex agglutination test (LAT), and enzyme-linked immunosorbent assay (ELISA) have been developed for the identification of BRV antigens (Dhama *et al.*, 2009). Moreover, methods based on molecular detection (hybridization test, reverse transcriptase (RT) - polymerase reaction (PCR), semi-nested or multiplex RT-PCR based on the VP4 and VP7 genes for genotyping, as well as real-time RT-PCR based on VP6) have also been developed by many researchers (Alfieri *et al.*, 2004; Chauhan and Singh, 1992; Cho *et al.*, 2013).

1.4.3.3. Laboratory Diagnosis of Bovine Coronavirus

The diagnosis of BCV in the laboratory is focused on the isolation or identification of pathogens, or serological methods, from the affected host by various methods. BCV is isolated from the feces, small intestinal tissues, and spiral colon tissue in various cell lines, including HmLu-1 cells (hamster lung cell line), Vero cell line (monkey kidney cell line), and HRT-18G (human rectal tumor cell line) (Table 8) (Hansa *et al.*, 2013; Jerez *et al.*, 2005; Saif, 2010). IFT, immunohistochemical staining (IHC), and ELISA have been used to detect BCV antigens. Meanwhile, molecular methods including RT-PCR, nested RT-PCR, and real-time RT-PCR have been widely used for the diagnosis of BCV (Boileau and Kapil, 2010; Saif, 2010). Among the molecular methods, the N protein gene is generally used for BCV

detection, while the S protein is used for phylogenetic analysis, epidemiological studies, and virus differentiation (Hodnik *et al.*, 2020). Moreover, for serological antibody detection, virus neutralization (VN) tests, hemagglutination inhibition (HI) tests, and ELISA have been widely used.

1.4.3.4. Laboratory Diagnosis of Bovine Viral Diarrhea Virus

Diagnostic methods for BVDV have been developed for the isolation of viruses, virus antigen detection, and specific antibody detection. Although any tissues from PI calves can be used for the virus isolation, blood samples, especially viable mononuclear cells, are most commonly used in BVDV sensitive cell lines, including the Madin-Darby Bovine Kidney (MDBK) cell line (bovine kidney cell line) (Dubovi, 2013) (Table 8). IHC has been used to detect the BVDV antigen and IHC using an ear notch showed 100% sensitivity (Cornish *et al.*, 2005). However, this method is limited to tissue samples. ELISA was also developed to detect BVDV antigens in various types of samples; however, the results from ELISA showed that specificity was affected by colostral antibodies in calves (Fux and Wolf, 2012). RT-PCR and real-time RT-PCR can detect BVDV antigens in various types of samples, including blood, feces, milk, saliva, and tissue samples. In addition to this, BVDV Types I and II can be differentiated by RT-PCR based on the 5' UTR gene (Lanyon *et al.*, 2014). Furthermore, specific BVDV antibody detection can be performed using rapid dot-blot enzyme immunoassay, agarose gel immunodiffusion,

microsphere-based immunoassay, ELISA, and VN test (Lanyon *et al.*, 2014).

1.4.3.5. Laboratory Diagnosis of Enterotoxigenic *E. coli*

As *E. coli* is a very common bacterium in the gastrointestinal tract, ETEC diagnosis focuses on the detection of virulence genes, including K99. Feces or fecal swabs are usually collected from diarrheic calves to detect virulence genes from *E. coli* colonies after they are cultivated either from samples or directly. For the cultivation of *E. coli*, samples from affected calves are inoculated onto MacConkey agar, blood agar, or eosin methylene blue agar (Table 8). From this, a single colony from the agar is collected and *E. coli* can then be identified by its biochemical properties (Ryu *et al.*, 2020a; Shams *et al.*, 2012). Whether it be from samples or *E. coli* colonies, virulence genes, including fimbrial adhesins (F5, F17, and F41), heat-labile toxins (LT), heat-stable toxins (STa and STb), and Shiga toxins 1 and 2, are usually targeted by specific PCR (Ryu *et al.*, 2020). K99 ETEC is determined by the detection of the K99 virulence gene by PCR.

1.4.3.6. Laboratory Diagnosis of *Salmonella* spp.

Diagnosis of *Salmonella* spp. in the laboratory is focused on the isolation or identification of pathogens. It should be considered in sample collection, before the cultivation of *Salmonella*, that *Salmonella* organisms are known to be easily outcompeted by other fecal gram negatives. Therefore, it is recommended that the

collected samples be stored in enrichment media, such as tetrathionate or selenite broth, and then transferred onto selective media (brilliant green or xylose lysine deoxycholate agar) (Table 8) (Barrow and Methner, 2013). Molecular detection methods, including PCR, are commonly used to identify *Salmonella* spp. from various sample types. These samples include milk, feces, bronchoalveolar lavage fluid, intestinal tissues (ileum, cecum, and colon), mesenteric lymph nodes, and the gall bladder (Holschbach and Peek, 2018). PCR is known to be more sensitive than bacterial culture; however, it is not always possible to perform subsequent serotyping (Nielsen, 2013).

1.4.3.7. Laboratory Diagnosis of *Cryptosporidium parvum*

The standard method for detecting *C. parvum* is considered to be the observation of oocysts in feces. Various methods have been developed to identify *C. parvum* oocytes, including Sheather's floatation, the formal ether concentration method, the formal ethyl acetate sedimentation technique, the Zeihl-Neelsen acid-fast staining method, as well as some negative staining methods using nigrosin, light green, malachite green, and carbol fuchsin (Table 8) (Ahmed and Karanis, 2018). Of these, fecal flotation with Sheather's sugar was found to be a more specific and sensitive method for the detection of oocysts (Rekha *et al.*, 2016). Immunoassay kits, immunofluorescence microscopy, and ELISA have been developed to detect antigens. In addition to these methods, PCR methods based on 18S ribosomal RNA have also been developed for this purpose (Garcia and Shimizu, 1997; Silverlås *et*

al., 2010).

1.4.3.8. Laboratory Diagnosis of *Eimeria* spp.

The diagnosis of *Eimeria* spp. is based on the observation of oocysts in feces. The presence of *Eimeria* spp. can be detected by a simple wet-mount technique; however, the fecal flotation method is more commonly used because this method can concentrate the oocysts and has been shown to be more sensitive than simple methods (Table 8) (Bangoura and Bardsley, 2020). Among various *Eimeria* spp., only a few species are known to be pathogenic and it is important to identify them at the species level to diagnose coccidiosis (Jolley and Bardsley, 2006). The other important factor in the diagnosis of *Eimeria* spp. is the quantification of oocyst shedding because it is related to the severity of the clinical signs. Several Ig antibodies in the maternal colostrum and the cow-calf operation that are specific to *Eimeria bovis* were investigated using ELISA and western blotting; however, this method was not useful for diagnosis (Fiege *et al.*, 1992).

Table 8. Isolation methods of pathogens associated with calf diarrhea

Category	Pathogens	Methods	Cell lines/Mediums	References
Viruses	Bovine rotavirus	Cell-culture	MA-104 cell line TF-104 cell line	Arnold *et al*., 2009 Dhama *et al*., 2009
	Bovine coronavirus	Cell-culture	HmLu-1 cell line Vero cell line HRT-18G cell line MDBK cell line	Hansa *et al.*, 2013; Jerez *et al.*, 2005; Saif, 2010
	Bovine viral diarrhea virus	Cell-culture	MDBK cell line	Dubovi, 2013
Bacteria	*E. coli*	Bacterial culture	MacConkey agar blood agar eosin methylene blue agar	Ryu *et al.*,2020a; Shams *et al.*, 2012
	Salmonella species	Bacterial culture	Brilliant green agar Xylose lysine deoxycholate agar	Holschbach and Peek, 2018
Protozoa	*Cryptosporidium parvum*	Fecal floatation	Sheather sucrose solution Zinc sulphate solution Saturated sodium chloride solution	Ahmed and Karanis, 2018; Rekha *et al.*, 2016
	Eimeria species	Fecal flotation	Sheather sucrose solution Saturated sodium chloride solution Potassium dichromate solution	Bangoura and Bardsley, 2020; Kim *et al*., 2018

1.5. Next Generation Sequencing

Next-generation sequencing (NGS) is a revolutionary, high-throughput technology that has transformed genomics research by enabling rapid, accurate sequencing of large volumes of DNA or RNA (Behjati and Tarpey, 2013). Unlike traditional Sanger sequencing, which processes a single DNA fragment at a time, NGS allows for the simultaneous sequencing of millions of DNA fragments. This significantly reduces sequencing time and cost, making large-scale genomic studies more accessible and affordable.

NGS has various applications across numerous fields, including human genetics, microbiology, plant biology, and veterinary science. It has proven instrumental in whole-genome sequencing, targeted gene sequencing, transcriptome analysis (RNA-seq), and epigenetic studies (Goodwin *et al*., 2016). NGS provides high-resolution genomic data, allowing researchers to obtain a comprehensive understanding of an organism's genetic makeup, gain insights into the molecular basis of diseases, explore gene expression patterns, and examine evolutionary relationships among species (Reuter *et al*., 2015).

The key advantages of NGS include high throughput, cost-effectiveness, flexibility, and high resolution (Behjati and Tarpey, 2013). These benefits have contributed to its widespread use and impact on various scientific disciplines, revolutionizing our understanding of genomics and advancing research in unprecedented ways.

1.5.1. Next Generation Sequencing in Veterinary Science

The application of NGS in veterinary science has far-reaching implications, from enhancing our understanding of animal genetics and disease mechanisms to improving diagnostic and therapeutic strategies. NGS has enabled researchers to delve deeper into the genetic architecture of various animal species. This has led to the identification of numerous genes and genetic variants associated with diseases, production traits, and other phenotypes. Uncovering the genetic basis of these traits not only improves our understanding of the underlying biology but also facilitates the development of targeted breeding programs and gene-editing strategies to enhance animal health, welfare, and productivity (Van Borm *et al*., 2015).

In addition, NGS enhanced disease diagnostics and surveillance in veterinary science. The rapid and accurate detection of pathogens is critical for the effective management of infectious diseases in animals. NGS-based approaches, such as metagenomics and targeted pathogen sequencing, have emerged as powerful tools for the identification and characterization of known and novel pathogens in a wide range of animal species (Kumar *et al*., 2019). These methods have proven particularly useful in monitoring and controlling the spread of emerging and re-emerging diseases, as well as in tracking the evolution of drug-resistant pathogens.

NGS technologies elucidated host-pathogen interactions. NGS such as RNA-seq, have provided unprecedented insights into the molecular mechanisms underpinning host-pathogen interactions (Pan *et al*., 2022). By elucidating the dynamic changes in gene expression and epigenetic regulation during infection,

researchers are now better equipped to develop novel therapeutic strategies and vaccines to combat infectious diseases in animals.

1.5.2. Pathogen Detection Using NGS technology

In acutely ill animals, the cause of suspected infections often remains undetermined, leading to delayed or insufficient treatment, and increased mortality and morbidity. The responsible agent can encompass a wide variety of both common and rare pathogens, including viruses, bacteria, fungi, and parasites. Conventional culture methods (i.e., growth in media) have limited success in recovering organisms due to early administration of broad-spectrum or prophylactic antimicrobial drugs, as well as the presence of fastidious or slow-growing organisms. Hypothesis-driven molecular testing, such as PCR, may involve multiple individual tests targeting specific organisms but may still miss rare pathogens or employ primers with mismatches to the microbial strain, decreasing detection sensitivity (Wilson *et al*., 2014). A hypothesis-free diagnostic approach with the potential to identify nearly any organism could lead to a significant shift in microbial diagnostic testing paradigms. However, a common concern with traditional testing methods is the restricted range of pathogens detected, often leaving clinicians with negative results and lingering doubts about whether the acute illness was caused by an infection for which no testing was performed.

One of the primary benefits of NGS is its unbiased sampling, which facilitates the broad detection of known and previously unknown pathogens, as well

as the potential discovery of new organisms (Chiu, 2013). Furthermore, NGS can be integrated with targeted approaches, such as utilizing conserved 16S ribosomal RNA and internal transcribed spacer sequences for universal bacterial and fungal identification, enabling species-level identification of these microorganisms (Salipante *et al*., 2014). Additionally, NGS can offer quantitative or semi-quantitative data regarding the concentration of organisms in a sample by counting sequenced reads, which proves valuable in polymicrobial samples or situations where multiple pathogens are involved in the disease process (Salipante *et al*., 2014).

1.5.3. Advantages and Challenges of NGS Technology for the Diagnosis of Bovine Diseases

Since the development of NGS technology, its use has expanded significantly in the diagnosis and study of various diseases in both humans and animals, including bovines. Notably, this technology presents several advantages over traditional diagnostic methods.

Firstly, NGS enables broad-spectrum pathogen detection, with the capacity to simultaneously identify a wide range of pathogens from a single test. This includes bacteria, viruses, parasites, and fungi. A demonstration of this capacity was the simultaneous detection of 43 common bovine and small ruminant bacterial, fungal, viral, and parasitic pathogens by NGS technology (Anis *et al*., 2018). Furthermore, NGS extends beyond identifying known pathogens to facilitate the discovery of

emerging or novel ones. By sequencing all genetic material present in a given sample, previously unknown or unexpected pathogens can be discovered. This was illustrated in the discovery of a novel pathogenic orbivirus in Kenya (Omoga *et al*., 2023) and a novel viral pathogen causing an epizootic outbreak of diarrhea in cattle in 2013 (Masuda *et al*., 2014).

Moreover, NGS technology supports precision medicine and treatment strategies. Detailed genetic data, provided by NGS, can be used to identify specific pathogen strains and their resistance genes. This information enables veterinarians to select the most effective drugs for treatment, thereby optimizing the therapeutic response. This approach was employed in the development of vaccines and treatments against many diseases, including bovine clinical mastitis and calf diarrhea (Beyi *et al*., 2021; Hoque *et al*., 2020).

However, the application of NGS technology in diagnosing bovine diseases also poses several challenges. As NGS generates a large volume of data, its handling and interpretation can be complex. Differentiating between pathogenic sequences, host sequences, and sequences from commensal or environmental organisms necessitates advanced bioinformatics capabilities. Moreover, associating these sequences with clinical relevance can be complex, requiring interdisciplinary expertise and careful interpretation.

Additionally, cost and accessibility pose considerable challenges. Despite recent cost reductions, NGS technology remains relatively expensive, considering not only the sequencing itself but also the associated costs of sample preparation,

bioinformatics analysis, and data storage. These expenses, combined with the required technical expertise, may render NGS inaccessible to some diagnostic labs or veterinary practices.

Finally, like any diagnostic test, NGS carries a risk of false positives and negatives. False positives may stem from contamination or sequencing errors, while false negatives may result if pathogen levels fall below the detection limit. Therefore, ensuring high-quality sample collection, preparation, and sequencing is paramount for reliable results. Further, since NGS in veterinary diagnostics is a relatively new field, standard protocols and validation measures are lacking. There is a pressing need for further validation of NGS in diagnosing bovine diseases, including studies to determine sensitivity, specificity, and predictive values compared to traditional methods.

Despite these challenges, the potential of NGS technology in diagnosing and understanding bovine diseases is substantial. The ongoing advancement in technology, reduction in cost, and development of bioinformatics solutions are likely to gradually overcome the current hurdles, further consolidating NGS as a central tool in veterinary diagnostics.

1.6. Prevention of Calf Diarrhea

1.6.1. Maintain Adequate Levels of Cow Nutrition

Even before the calf is born, calf diarrhea can be prevented by maintaining

the health of the maternal cows. The severity of the incidences of calf diarrhea, and overcoming this disease, is related to the health of these cows. The nutritional status of maternal cows during early gestation can affect calf health. Furthermore, good nutritional status has been associated with good quality of oocytes, embryo survival, placental angiogenesis, and cotyledon weight, which are related to calf health (Vonnahme *et al.*, 2007). In addition, adequate nutritional status in late gestation is known to be related to an increase in birth weight. As birth weight is positively correlated with calf growth rate up to the weaning period and live weight at weaning, it is important to supply adequate levels of nutrition to maternal cows.

Dystocia is a difficult birth process that cannot be completed without assistance. Abnormal nutrition status of cows can cause dystocia, which can affect calf health, with cows that experienced dystocia showing more body condition score than cows without dystocia. Dystocia can cause hypoxia, acidosis, and congestion of the head and tongue, which can be related to prolonged colostrum ingestion, resulting in a higher risk of calf diarrhea. Calves with dystocia showed a 1.44 relative risk compared to calves born without assistance (Bendali *et al.*, 1999).

1.6.2. Controlling the Environment

Calves cannot effectively regulate their body temperature. As a result, many weather conditions, including low temperature, high humidity, wind, and rain, can affect a calf's health (Larson and Tyler, 2005). In a negative health environment,

calves can suffer from negative energy metabolism, which can impair the immune system. This results in calf diarrhea. Therefore, it is important to rear calves, without the effect of the above-mentioned climate conditions, by using proper calf pens and heat lamps.

Controlling contamination is important not only to control the climatic conditions, but also to prevent calf diarrhea. To prevent contamination from the environment, providing maternity areas with clean and dry bedding; cleaning and disinfecting the vehicle for transportation of neonatal calves; maintaining good hygiene when dealing with colostrum; pasteurizing milk; minimizing contact with other groups of cattle or calves; and maintaining effective measures of biosecurity for equipment, fomites, and personnel are suggested (Maunsell and Donovan, 2008).

1.6.3. High Quality Colostrum and Vaccination

Maintaining high-quality colostrum is a key factor in preventing calf diarrhea. The importance of colostrum to calves is related to the fact that calves get their antibodies only from colostrum. This is because the structure of bovine placenta does not allow the passive transfer of antibodies to the fetus. Therefore, the amount of immunoglobulins absorbed by calves depends on the quality of the colostrum (Arsenopoulos *et al.*, 2017). While colostrogenesis is not-well studied, it is known that antibodies in the blood of pregnant cows slowly accumulate in the mammary glands before calving, aided by the neonatal Fc receptor. These antibodies are then released in high concentrations, along with other components of colostrum,

immediately after calving (Mayer *et al.*, 2005). Colostrum containing more than 50 g/L of immunoglobulin is considered to be of good quality and it is recommended that calves are fed a total of 150 g to 200 g of IgG (Chigerwe *et al.*, 2008).

Another way to prepare high-quality colostrum to prevent calf diarrhea is to develop specific antibodies against the pathogens that cause calf diarrhea by vaccination and containing enough concentrations of antibodies to prevent infection in colostrum. During the first 24 hours of life, the enterocytes of the small intestine absorb various macromolecules, including Ig, via pinocytosis. The absorbed Ig is then transported across the intestinal barrier into the bloodstream and is secreted into the intestinal lumen to protect against pathogens (Mayer *et al.*, 2002). In this process, specific anti-pathogen antigens derived from maternal cows can affect the severity of infections caused by pathogens that cause calf diarrhea.

Chapter I

Hematology and Serum Biochemistry in Korean Indigenous (*Bos taurus coreanae*) Calves with Diarrhea

Abstract

This study was conducted to identify the useful blood variables in diagnosing calf diarrhea in KIC and good indicators for calf diarrhea. In 530 KIC, fecal scores were recorded on a scale of 0 to 3, and blood samples were collected and analyzed for hematology, serum biochemistry, and acute phase proteins. Among the blood variables, 16 blood variables showed significant differences ($p < 0.01$) according to fecal scores. After reference intervals of these 16 blood variables were calculated, the distributions of calves by calculated reference intervals showed a significant difference ($p < 0.001$) and linear associations ($p < 0.001$) in blood urea nitrogen (BUN), glucose (GLU), blood sodium concentration (Na), blood potassium

concentration (K), fibrinogen (Fib), and haptoglobin (Hp). Of 6 blood variables, the optimal cut-off values were calculated for BUN, K, Fib, and Hp, and the area under the curve was 0.5 or more: BUN (9.5 mg/dL, AUC: 0.623), K (5.8 mmol/L, AUC: 0.599), Fib (650.0 mg/dL, AUC: 0.706), and Hp (12.5 mg/dL, AUC: 0.847). These findings could be useful in diagnosing calf diarrhea in KIC.

Keywords: Korean indigenous calves, calf diarrhea, hematology, serum biochemistry, acute phase proteins.

1.1. Introduction

Calf diarrhea is one of the most prevalent diseases in cattle industries. It has affected the morbidity and mortality of neonatal calves and their growth performances and has caused worldwide economic loss (Cho and Yoon, 2014). In diarrhea, calves lose their body fluids, electrolytes, and other substances from the body, which leads to electrolyte imbalance. This, in turn, causes the concentration of ions and other substances remaining in the fluid to rise, which increases the osmotic pressure of the extracellular fluid. This mechanism results in electrolyte imbalances and metabolic changes (Dratwa-Chałupnik *et al*., 2012).

To diagnose calves affected by diarrhea, these metabolic changes should be well-evaluated for further treatment. Among various methods, blood analysis is considered to be one of the most useful methods for diagnosing many organ and systemic diseases in veterinary science (Ježek *et al*., 2006; Roland *et al*., 2014). During analysis, data obtained from the healthy and diseased animals can provide an insight into health and metabolic status. Information on the blood variables of various cattle species has been well described by previous studies; however, there is not enough data regarding Korean indigenous cattle (*Bos taurus coreanae*), especially with regard to calves (Cherdthong *et al*., 2014; George *et al*., 2010; Herd *et al*., 2019, Lee *et al*., 2015).

Measuring the level of acute phase proteins (APPs) in blood is also considered an effective method for evaluating the condition of the host. The acute

phase response is a reaction of the host to homeostatic disturbances caused by infections, tissue injuries, neoplastic growth, or immunological disorders, and the concentration of APPs in blood is altered accordingly (Jawor and Stefaniak, 2011). In addition, as inflammation is not always followed by an increase in leukocyte population in cattle, APPs are considered to be a sensitive marker that can help evaluate the status of the host. Although various APPs have been reported so far, many studies considered Fib, Hp, and SAA as major APPs in cattle (Jawor and Stefaniak, 2011). However, there have been a lack of research about the relationship between APPs and calf diarrhea.

Many reports have suggested that hematological and serum biochemistry values are different from various factors including species or growth stages; therefore, it is important to analyze the hematology and serum biochemistry for each species of animals before diagnosing a disease using blood test results (Panousis *et al*., 2018; Tóthová *et al*., 2016). However, because blood tests show different results depending on the test machines or methods, accurate analysis could be performed for the diagnosis of the disease only if the analyzer has its own reference intervals (RI) for the machines (Jain *et al*., 2009). Therefore, this study was conducted to identify useful blood variables in diagnosing calf diarrhea, especially in KIC.

1.2. Materials and Methods

Ethical statement

All procedures were performed according to ethical guidelines for the use of animal samples, as approved by the Institutional Animal Care and Use Committee (IACUC) in Jeonbuk National University (IACUC No. JBU 2016-00026).

Farm characteristics

Farm information (location, province, size, type, animal taker, and history of vaccination) was described in Table 9. The size of farms was divided into the number of raised cattle in farms (small, less than 100, medium, 101~300, large, more than 301). Farm type was described litter barn & conventional or mooring & organic. Animal taker was described the people who raised cattle: farm owners or employees. History of vaccination was recorded if raised dams were vaccinated, regarding to calf diarrhea.

Sample collection

Ten farms in five provinces (Gangwon-do, Chungcheongnam-do, Jeollabuk-do, Gyeongsangbuk-do, and Gyeongsangnam-do) in the ROK were subjected for this study. Blood was collected from calves up to 60 days of age in the 10 farms during 2016~2017. Age, farm, and fecal scores were recorded. Ten mL blood was collected from the jugular vein of each calf. Three mL of whole blood

was stored in two K2 ethylene diamine tetraacetic acid (EDTA) tubes (BD vacutainer®) at 4°C, and 4 mL of blood was allowed to clot at room temperature in serum separating tube (SST™) (BD vacutainer®) and immediately transported to the laboratory. The fecal consistency scoring system is a 4-level scoring system based on the degree of fluidity of the feces (score 0, normal; score 1, semi-formed; score 2, loose but bedding; score 3, watery) created by the University of Wisconsin-Madison School of Veterinary Medicine (McGuirk, 2008) (Fig. 4).

Laboratory procedures

The SST tube and one K2 EDTA tube were centrifuged at 3000 × g for 30 min to separate the serum and plasma, respectively. Complete blood count including red blood cell (RBC), HCT, Hb, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte (RETIC), platelet (PLT), white blood cell (WBC), neutrophil (NE), lymphocyte (LYM), monocyte (MO), eosinophil (EO), and basophil (BA) in the whole blood and blood chemistry [Total serum protein (TP), albumin (ALB), BUN, GLU, blood sodium concentration (Na), blood potassium concentration (K), and blood chloride concentration (Cl)) using serum were analyzed with an automatic blood analyzer (IDEXX ProcyteDx, IDEXX Laboratories, Westbrook, ME). Commercial enzyme-linked immunosorbent assay (ELISA) kits (PHASE™ RANGE multispecies SAA ELISA kit, Tridelta Development Ltd., County Kildare, Ireland; PHASE™ RANGE Haptoglobin kit, Tridelta Development Ltd., County Kildare,

Ireland) were used to evaluate SAA and Hp in serum, following the manufacturer's instruction. Protein levels in the plasma and serum samples were calculated using an optical clinical refractometer (MASTER-SUR/Nα, ATAGO CO., LTD., Tokyo, Japan). Estimated Fib level was calculated by subtracting the plasma protein from the serum protein.

Statistical analysis

All blood variables were divided by fecal scores. All variables were compared by fecal scores using one-way analysis of variance (ANOVA) and Tukey's test for post hoc comparisons. The P values < 0.05 were considered statistically significant. RI was calculated using the Reference Value Advisor (v.2.1) freeware, a set of macroinstructions for Microsoft Excel (Geffré *et al*., 2011), according to the guidelines recommended by the American Society for Veterinary Clinical Pathology (Friedrichs *et al*., 2012). The validity of blood variables for the diagnosis of calf diarrhea was decided when the distribution of calves according to RI and fecal consistency showed significant difference in the linear-by-linear association and Pearson's chi-squared test. Next, the receiver operating characteristic (ROC) curve and the area under the curve (AUC) for fecal score 3 were calculated to determine whether blood variables could be effectively used for the prognosis of calf diarrhea. For the interpretation of AUC, $0.5 < \text{AUC} \leq 0.7$ was considered to indicate less diagnostic accuracy and $0.7 < \text{AUC} \leq 0.9$ was considered moderate diagnostic accuracy (Pak and Oh, 2016). All statistical analyses were performed using SPSS

(Version 25.0; IBM Corp., Armonk, NY), and all graphical analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA).

1.3. Results

Sample collection

Blood samples were collected from 530 KIC at 3 to 60 days of age (median: 29 days old) raised on 10 farms. A total of 530 calves were categorized into 145 calves in fecal score 0, 185 calves in fecal score 1, 114 calves in fecal score 2, 86 calves in fecal score 3 (mean: 1.19).

Comparison of blood variables according to the fecal scores

To determine the blood variables to calculate RI, all blood results were compared according to the fecal scores. The mean and standard deviation of blood variables for each fecal score are described in Table 10. Among the variables, RBC, Hb, MCHC, RETIC, WBC, LYM, MO, Alb, BUN, GLU, Na, K, Cl, Fib, SAA, and Hp showed significant differences ($p < 0.05$) according to the fecal scores (Table 10); thus, additional analysis was performed.

Comparison of the distribution of KIC according to RI and fecal scores

To compare the distribution of 530 KIC according to RI in blood variables shown significantly different by fecal scores, the RI for each blood variable was calculated for blood variables that were significantly different according to fecal scores. The described statistics and calculated 95% RI with 90% confidence interval

for RBC, Hb, MCHC, RETIC, WBC, LYM, MO, Alb, BUN, GLU, Na, K, Cl, Fib, SAA, and Hp in 145 KIC with fecal score 0 are presented in Table 11. After RI calculation, the distribution of 530 KIC according to fecal scores and blood variables that were significantly different by fecal scores was investigated (Table 12). Thus, the distributions of calves showed significant difference and linear associations in terms of BUN, GLU, Na, K, Fib, and Hp. The number of calves that showed lower than RI was increased in GLU and Na as fecal scores increased. In contrast, the number of calves that showed higher than RI was increased in BUN, K, Fib and Hp, as fecal scores increased.

Determination of optimal cut-off values for calf diarrhea in KIC

To determine the valuable blood variables and their optimal cut-off values of calf diarrhea, ROC analysis was performed. The results of ROC curve analysis, sensitivity-specificity analysis, and determination of cut-off values of BUN, Na, GLU, K, Fib, and Hp are described in Table 13. The predictive accuracy of BUN was low (AUC: 0.623, 95% CI 0.554~0.692, $p < 0.001$) at a BUN cut-off value of 9.5 mg/dL, and the sensitivity and specificity in predicting calf diarrhea were 60.0% and 56.1%, respectively. The predictive ability of GLU was poor (AUC: 0.271, 95% CI 0.207~0.335, $p < 0.001$), and at a GLU cut-off value of 83.5 mg/dL, the sensitivity and specificity in predicting calf diarrhea were 30.2% and 30.6%, respectively. The predictive ability of Na was poor (AUC: 0.366, 95% CI 0.259~0.392, $p < 0.001$) and at a Na cut-off value of 137.5 mmol/L, and the sensitivity and specificity in

predicting calf diarrhea were 39.8% and 37.1%, respectively. The predictive ability of K was low (AUC: 0.599, 95% CI 0.526~0.672, $p < 0.001$), at a K cut-off value of 5.8 mmol/L, the sensitivity and specificity in predicting calf diarrhea were 58.3% and 54.2%, respectively. The predictive ability of Fib was moderate (AUC: 0.706, 95% CI 0.644~0.768, $p < 0.001$), at a Fib cut-off value of 650.0 mg/dL, the sensitivity and specificity in predicting calf diarrhea were 68.6% and 61.9%, respectively. The predictive ability of Hp was moderate (AUC: 0.847, 95% CI 0.801~0.893, $p < 0.001$), at a Hp cut-off value of 12.5 mg/dL, the sensitivity and specificity in predicting calf diarrhea were 74.4% and 75.9%, respectively.

1.4. Discussion

In calf diarrhea, various metabolic and inflammatory changes occur in the body of the calf, so it is important to identify these changes during diagnosis (Seifi *et al*., 2006). In this study, among the various methods for evaluating these changes, the analyses of hematology, serum biochemistry, and some acute phase proteins were performed to identify useful blood variables in the diagnosis of calf diarrhea.

RBC, Hb, MCHC, RETIC, WBC, MO, Alb, BUN, GLU, Na, K, Cl, Fib, SAA, and Hp showed significant difference according to their fecal scores. The blood variables related to CBC panels (RBC, Hb, MCHC, RETIC, WBC, MO) showed greater differences than was observed in other reports, however, these differences were considered to be normal physiological changes in young calves (Lee *et al*., 2015; Panousis *et al*., 2018). Moreover, although Fib and SAA in this study were higher than those reported in previous studies, similar Fib and SAA values were also reported in KIC of similar ages (Choi *et al*., 2021; Kang *et al*., 2020).

In the RI calculation, many factors should be considered including the number of samples (Friedrichs *et al*., 2012). The number of samples is crucial because of the accuracy of calculation, statistical significance, reducing bias, and confidence in the results and the RI can be calculated using a nonparametric method that is statistically sufficiently significant when there are at least 120 reference samples guidelines recommended by the American Society for Veterinary Clinical

Pathology (Friedrichs *et al*., 2012). The number of calves for RI calculation in this study was relatively small to represent the exact RI of blood variables in KIC; nevertheless, further analysis was conducted because the RI in this research was considered to be reliable.

After comparing the RI of blood variables with fecal scores, BUN, GLU, Na, K, Cl, Fib, and Hp were found to be useful blood variables in calf diarrhea. In this research, the distribution rates of 530 KIC showing higher BUN than RI (4.8% to 34.9%) significantly increased with increasing fecal scores from 0 to 3 ($p < 0.001$). In particular, the percentage of calves with fecal score 3 showing azotemia (34.9%) was more than 7.2 times higher than that of calves with fecal score 0 showing azotemia (4.8%). Higher BUN was commonly reported in calf diarrhea with dehydration and BUN in diarrheic calves that died was significantly higher ($p < 0.01$) than in diarrheic calves that recovered in a previous report (Smith and Berchtold, 2014). Furthermore, BUN was significantly influenced by physical activity, dehydration status, and prognosis of calf diarrhea ($p < 0.001$) (Lee *et al*., 2020). These differences could be attributed to the severity of calf diarrhea. Consistent with these results, BUN showed predictive ability in this research, which could prove useful for the prognosis of calf diarrhea in terms of fecal scores.

Significant changes in GLU levels according to fecal scores were detected in this research. With increasing fecal scores from 0 to 3, the distribution rates of 530 KIC showing lower GLU than RI was significantly increased (3.4% to 26.7%) ($p < 0.001$). The percentage of calves with fecal score 3 showing hypoglycemia (26.7%)

was more than 7.8 times higher than that of calves with fecal score 0 showing hypoglycemia (3.4%). Hypoglycemia has been reported in diarrheic calves, especially those that were weak, lethargic, or comatose and usually occurs in the terminal stages of the disease (Tsukano *et al*., 2018). Severe hypoglycemia in calf diarrhea was associated with poor survival rates (20.6%), and calves with severe hypoglycemia (less than 40 mg/dL) were extremely dehydrated (Smith and Berchtold, 2014). However, GLU showed no predictive ability in this study, which was also reported in other studies that hypoglycemia showed 3.09 times higher mortality in calves with diarrhea; however, the predictive ability of GLU was poor (AUC=0.384) according to their ROC analysis (Tsukano *et al*., 2018).

Significant changes in Na and K levels according to fecal scores were detected in the present study. With increasing fecal scores from 0 to 3, the distribution rates of 530 KIC showing lower Na than RI was significantly increased (3.4% to 26.7%) ($p < 0.001$), and the percentage of calves with fecal score 3 showing hyponatremia (26.7%) was more than 7.8 times higher than that of calves with fecal score 0 (3.4%). Additionally, with increasing fecal scores from 0 to 3, the rates of KIC showing higher K than RI was significantly increased (3.4% to 22.1%) ($p < 0.001$), and the percentage of calves with fecal score 3 showing hyperkalemia (22.1%) was 6.5 times higher than that of calves with fecal score 0 showing hyperkalemia (3.4%). Electrolytes could vary considerably in calves with diarrhea. Many previous studies reported various changes (lower to higher RI) in electrolytes (Na, K, Cl) during calf diarrhea (Lee *et al*., 2020; Sayers *et al*., 2016; Trefz *et al*., 2017). These

differences were attributed to the severity, duration, and causes of calf diarrhea (Dratwa-Chałupnik *et al*., 2012). K showed predictive ability while Na showed no predictive ability in calf diarrhea in this study. Hyperkalemia was most closely associated with dehydration and weakly correlated with venous blood pH levels (Constable and Grünberg, 2013). Moreover, K was more strongly associated with calf diarrhea than Na in other reports (Trefz *et al*., 2013).

The method to measure Fib in this research was based on the fact that serum does not contain Fib and clotting factors which are the proteins found in plasma. Therefore, plasma protein levels subtracted by serum protein levels would be indirectly correlated to the amount of Fib, which is known as an acute phase protein. The distribution rates of 530 KIC showing higher Fib (2.8% to 54.7%) than RI significantly increased with increasing fecal scores from 0 to 3 ($p < 0.001$), and the percentage of calves with fecal score 3 showing hyperfibrinogenemia (54.7%) was 19.5 times higher than that of calves with fecal score 0 showing hyperfibrinogenemia (2.8%). The distribution rates of 530 KIC showing higher Hp (1.4% to 58.1%) than RI significantly increased with increasing fecal scores from 0 to 3 ($p < 0.001$), and the percentage of calves with fecal score 3 showing hyperhaptoglobinemia (58.1%) was more than 41.5 times higher than that of calves with fecal score 0 showing hyperhaptoglobinemia (1.4%). To our knowledge, this study is the first to measure the RI of acute phase proteins and its application to calf diarrhea in KIC. In addition, Fib and Hp showed moderate predictive ability. These results support the fact that Hp and Fib are major acute phase proteins in cattle. Many

researchers have reported the usefulness of quantifying these proteins when monitoring animal health, especially to determine the inflammatory status of the animal (El-Deeb *et al*., 2020; Jawor and Stefaniak, 2011)

In the ROC analysis, the predictive abilities of BUN, GLU, Na, K, Fib, and Hp was evaluated. The acceptable predictive ability of BUN (AUC: 0.636), K (AUC: 0.617), Fib (AUC: 0.706), and Hp (AUC: 0.847) was evaluated, and their optimal cut-off values were calculated as follows: BUN (9.5 mg/dL), K (5.8 mmol/dL), Fib (650.0 mg/dL), and Hp (12.5 mg/dL). BUN and K were valuable prognostic indicators for predicting the fate of diarrheic calves according to a previous study, which was consistent with our results (Seifi *et al*., 2006). Furthermore, although studies evaluating the prognostic value of Hp and Fib are lacking, many studies have reported increased Hp in calf diarrhea and other calf diseases (El-Deeb *et al*., 2020; Hajimohammadi *et al*., 2013). The findings in this study would be helpful in the treatment and prognostic values of calf diarrhea. However, this study had a limitation that actual treatment of calf diarrhea was not performed, so further studies might need to evaluate the cut-off values of BUN, K, Fib, and Hp in the actual treatment and prognosis evaluation of calf diarrhea.

1.5 Conclusions

In this study, BUN, GLU, Na, K, Fib, and Hp showed significant difference and linear associations in fecal scores. Furthermore, after ROC analysis, the optimal cut-off values of BUN, K, Fib, and Hp were calculated. Although many previous studies showed CBC, serum biochemistry, APPs and pro-inflammatory cytokines in diarrheic and non-diarrheic calves, specific values of blood variables were not provided because of not enough number of calves. In this study, blood variables that could be useful for calf diarrhea, and ROC analysis to understand the diagnostic and prognostic power of some blood variables, and their optimal cut-off values were provided in large number of KIC. These findings would be useful for field veterinarians and animal caretakers in diagnosing and predicting the prognosis of calf diarrhea in KIC.

Table 9. The description of 10 Korean indigenous cattle farms investigated for calf diarrhea in 2016~2017

Farms	Provinces	Locations	Size of farms*	Farm types	Animal care takers	Vacci-nation againt calf diarrhea	No. of samples (calves)				
							Fecal scores				Total (n=530)
							0 (n=145)	1 (n=185)	2 (n=114)	3 (n=86)	
1	Gangwon-do	Heongseong	M	LC	Owner	No	0	12	4	1	17
2	Chungcheong nam-do	Asan	L	LC	Employee	No	29	39	21	16	105
3	Gyeongsang buk-do	Yeongju	M	LC	Owner	No	44	58	39	31	172
4	Gyeongsang buk-do	Mungyeong	M	LC	Owner	No	12	9	4	1	26
5	Gyeongsang buk-do	Sangju	S	LC	Owner	No	2	4	2	1	9
6	Jeollabuk-do	Wanju	M	LC	Owner	No	13	14	5	3	35
7	Jeollabuk-do	Iksan	S	LC	Owner	No	6	1	0	1	8
8	Jeollabuk-do	Samnye	M	LC	Owner	No	25	30	17	12	84
9	Jeollabuk-do	Gimje	M	LC	Owner	No	4	15	22	20	61
10	Gyeongsang nam-do	Sancheong	S	MO	Owner	No	10	3	0	0	13

No., Number; *Farm size: S, small, less than 100 cows; M, medium, 101~300 cows; L, large, more than 301 cows. LC, litter barn and conventional; MO, mooring and organic.

Table 10. The mean and standard deviation description of blood parameters of 530 Korean indigenous calves less than 60 days according to fecal scores

Parameters	Fecal scores				F value	P value
	0 (n=145)	1 (n=185)	2 (n=114)	3 (n=86)		
RBC (10^6/μL)	10.13 ± 1.95[ab]	9.74 ± 1.84[a]	10.12 ± 2.09[a]	10.66 ± 2.19[b]	5.156	0.002
HCT (%)	35.86 ± 8.22[a]	34.21 ± 8.38[a]	35.46 ± 8.42[a]	36.21 ± 8.09[a]	1.914	0.126
Hb (g/dL)	11.50 ± 2.26[ab]	10.95 ± 2.02[a]	11.53 ± 2.48[ab]	12.00 ± 2.15[b]	5.511	0.001
MCV (fL)	35.36 ± 4.11[a]	34.96 ± 5.01[a]	35.47 ± 5.64[a]	34.34 ± 5.74[a]	0.978	0.403
MCH (pg)	11.42 ± 1.29[a]	11.42 ± 1.81[a]	11.53 ± 1.55[a]	11.40 ± 1.64[a]	0.190	0.903
MCHC (g/dL)	32.42 ± 2.52[a]	32.41 ± 3.76[a]	32.98 ± 2.84[ab]	33.64 ± 3.47[b]	3.399	0.018
RETIC (10^3/μL)	2.10 ± 2.10[a]	3.31 ± 4.25[b]	2.15 ± 1.69[ab]	2.52 ± 2.78[ab]	4.867	0.002
WBC (10^3/μL)	10.75 ± 3.29[a]	11.00 ± 4.15[a]	11.76 ± 5.14[ab]	12.60 ± 8.08[b]	3.100	0.026
NE (10^3/μL)	4.48 ± 2.57[a]	5.09 ± 3.17[a]	5.37 ± 4.18[a]	5.64 ± 6.08[a]	1.900	0.129
LY (10^3/μL)	5.20 ± 1.71[a]	4.93 ± 1.93[a]	5.33 ± 1.84[a]	5.78 ± 4.19[b]	2.849	0.037
MO (10^3/μL)	0.90 ± 0.64[ab]	0.73 ± 0.60[a]	0.92 ± 0.58[ab]	0.99 ± 0.95[b]	4.171	0.006
EO (10^3/μL)	0.17 ± 0.21[a]	0.17 ± 0.31[a]	0.12 ± 0.19[a]	0.15 ± 0.27[a]	1.487	0.217
BA (10^3/μL)	0.03 ± 0.10[a]	0.01 ± 0.03[a]	0.02 ± 0.10[a]	0.02 ± 0.05[a]	1.206	0.307
PLT (10^3/μL)	711.26 ± 331.11[a]	711.18 ± 298.16[a]	793.97 ± 292.34[a]	754.00 ± 312.73[a]	2.547	0.055

Values indicated by distinct letters differ significantly (Tukey HSD test, $p < 0.05$).

Table 10. The mean and standard deviation description of blood parameters of 530 Korean indigenous calves less than 60 days according to fecal scores (Continued)

Parameters	Fecal scores				F value	P value
	0 (n=145)	1 (n=185)	2 (n=114)	3 (n=86)		
Total (Serum) Protein (g/dL)	6.02 ± 0.75[a]	5.93 ± 0.76[a]	6.13 ± 0.69[a]	5.90 ± 0.77[a]	1.306	0.272
Alb (g/dL)	2.71 ± 0.33[ab]	2.61 ± 0.28[a]	2.75 ± 0.34[b]	2.74 ± 0.42[b]	5.179	0.002
BUN (mg/dL)	8.86 ± 3.48[a]	10.83 ± 9.53[a]	15.89 ± 16.35[b]	15.86 ± 13.93[b]	12.440	0.000
GLU (mg/dL)	95.73 ± 15.79[a]	87.64 ± 18.21[b]	88.72 ± 15.86[b]	76.12 ± 21.70[c]	22.117	0.000
Na (mmol/L)	139.23 ± 4.09[a]	138.60 ± 5.00[ab]	136.96 ± 7.19[b]	133.80 ± 7.89[c]	16.801	0.000
K (mmol/L)	5.66 ± 0.67[a]	5.72 ± 0.66[a]	6.22 ± 1.32[b]	6.51 ± 1.78[b]	15.953	0.000
Cl (mmol/L)	99.91 ± 3.57[a]	99.38 ± 2.75[ab]	98.63 ± 3.65[bc]	97.54 ± 4.47[c]	8.618	0.000
Fibrinogen (mg/dL)	546.20 ± 106.08[a]	588.11 ± 224.02[a]	753.51 ± 288.16[b]	802.33 ± 289.81[b]	35.113	0.000
SAA (mg/L)	168.44 ± 123.35[a]	256.84 ± 145.96[b]	230.94 ± 127.99[b]	250.74 ± 142.61[b]	12.105	0.000
Haptoglobin (mg/dL)	5.86 ± 6.65[a]	7.67 ± 11.78[a]	15.57 ± 9.662[b]	31.19 ± 22.91[c]	76.631	0.000

Values indicated by distinct letters differ significantly (Tukey HSD test, $p < 0.05$).

Table 11. Reference intervals (RI) for blood parameters of 145 healthy Korean indigenous calves

Parameters	Descriptive statistics		95% RI		RI in calves	References
	Mean±SD	Median	Lower limits & CI (90%)	Upper limits & CI (90%)		
RBC (10^6/μL)	10.13±1.95	10.31	6.25 (5.85~6.68)	13.96 (13.52~14.39)	6.2-11.9	Roadknight *et al*., 2021
Hb (g/dL)	11.50±2.26	11.90	6.95 (6.47~7.44)	15.95 (15.43~16.44)	5.6-13.8	
MCHC (g/dL)	32.42±2.52	0.80	27.25 (26.53~28.04)	37.52 (36.75~38.31)	30.2-37.7	
RETIC (10^3/μL)	2.10±2.10	1.80	0.00 (0.00~0.00)	9.54 (6.80~12.50)	0-8.5	
WBC (10^3/μL)	10.75±3.29	10.24	4.07 (3.43~4.73)	17.23 (16.23~18.30)	3.84-19.55	
LYM (10^3/μL)	5.20±1.71	4.80	2.47 (1.69~2.69)	9.37 (8.41~10.50)	1.43-8.16	
MO (10^3/μL)	0.90±0.64	0.85	0.01 (0.00~0.02)	2.10 (1.83~5.10)	0.02-1.25	
Albumin (g/dL)	2.71±0.33	2.70	2.03 (1.95~2.12)	3.37 (3.26~3.49)	2.7-3.6	
BUN (mg/dL)	8.86±3.46	8.01	3.01 (1.01~4.03)	15.72 (14.74~16.82)	4.48-21.56	
GLU (mg/dL)	95.73±15.79	96.50	64.86 (61.59~68.25)	126.93(123.35~130.36)	50.4-124.2	
Na (mmol/L)	139.23±40.9	139.00	131.15(129.82~132.53)	147.22(145.75~148.94)	130-148	
K (mmol/L)	5.66±0.67	5.60	4.32 (4.19~4.46)	7.02(6.80~7.25)	4.75-6.75	
Cl (mmol/L)	99.91±3.57	100.00	92.81(91.57~94.12)	106.89(105.69~108.19)	96-106	
Fibrinogen (mg/dL)	546.20±106.08	500.0	328.52 (303.14~354.23)	766.12 (738.70~795.44)	0-590.0	Seppä-Lassila *et al*., 2013
SAA (mg/L)	168.44±123.35	143.67	0.47 (0.00~14.37)	526.19 (389.15~669.40)	0-178.0	
Haptoglobin (mg/dL)	5.86±6.65	5.00	0.00(0.00~0.00)	24.01 (16.31~32.02)	0-19.6	

CI, confidence interval; SD, Standard Deviation.

Table 12. Average of hematology and serum biochemistry according to the fecal score of 530 Korean indigenous calves

Parame-ters	RI groups	Fecal scores								P value	
		0 (n=145)		1 (n=185)		2 (n=114)		3 (n=86)			
		Mean±SD	No. of calves	Mean±SD	No. of calves	Mean±SD	No. of calves	Mean±SD	No. of calves	Pe	Li
RBC (10^6/μL)	Lower RI	5.01±0.95	2 (1.4%)	5.29±0.75	5 (2.7%)	5.22±1.04	4 (3.6%)	-	0 (0.0%)		
	Within RI	10.11±1.76	140 (96.6%)	9.79±1.60	177 (95.7%)	10.09±1.74	107 (93.9%)	10.43±1.82	80 (93.0%)	0.117	0.076
	Higher RI	14.59±0.44	3 (2.1%)	14.49±0.36	3 (1.6%)	15.25±0.81	3 (2.6%)	15.15±0.94	6 (7.0%)		
Hb (g/dL)	Lower RI	5.80±0.28	2 (1.4%)	6.50±0.34	4 (2.2%)	5.74±0.93	5 (4.4%)	-	0 (0.0%)		
	Within RI	11.49±2.09	140 (96.6%)	11.03±1.88	180 (97.3%)	11.55±1.95	106 (93.0%)	12.02±1.95	85 (98.8%)	0.258	0.978
	Higher RI	16.17±0.12	3 (2.1%)	16.50	1 (0.5%)	18.17±1.54	3 (2.6%)	19.30	1 (1.2%)		
MCHC (g/dL)	Lower RI	25.13±0.59	3 (2.1%)	23.82±6.53	12 (6.5%)	24.73±1.40	3 (2.6%)	26.70±0.14	2 (2.3%)		
	Within RI	32.31±1.95	136 (93.8%)	32.56±1.93	162 (87.6%)	32.86±2.04	105 (92.1%)	32.76±1.93	74 (86.0%)	0.077	0.082
	Higher RI	38.60±1.57	6 (4.1%)	39.76±2.35	11 (5.9%)	39.73±1.51	6 (5.3%)	41.16±1.88	10 (11.6%)		
RETIC (10^3/μL)	Lower RI	-	0 (0.0%)	-	0 (0.0%)	-	0 (0.0%)	-	0 (0.0%)		
	Within RI	1.90±1.66	136 (93.8%)	2.44±1.97	155 (83.8%)	2.19±1.69	108 (94.7%)	2.10±1.99	82 (95.3%)	0.001	0.244
	Higher RI	11.00±1.45	9 (6.2%)	16.92±6.61	30 (16.2%)	13.9±1.23	6 (5.3%)	12.20±1.13	4 (4.7%)		
WBC (10^3/μL)	Lower RI	3.63	1 (0.7%)	3.43±0.32	2 (1.1%)	-	0 (0.0%)	-	0 (0.0%)		
	Within RI	10.43±2.74	138 (95.2%)	10.24±3.01	169 (91.4%)	10.51±3.12	103 (90.4%)	9.81±2.79	72 (83.7%)	0.051	0.003
	Higher RI	19.43±1.89	6 (4.1%)	20.30±4.03	14 (7.6%)	23.45±5.77	11 (9.6%)	26.69±11.16	14 (16.3%)		
LYM (10^3/μL)	Lower RI	2.04±0.50	2 (1.4%)	1.94±0.42	16 (8.6%)	1.72±0.15	3 (2.6%)	2.13	1 (1.2%)		
	Within RI	5.03±1.40	136 (93.8%)	5.07±1.42	164 (88.6%)	5.11±1.29	105 (92.1%)	5.11±1.49	79 (91.9%)	0.009	0.554
	Higher RI	9.49±0.60	7 (4.8%)	11.08±4.26	5 (2.7%)	10.26±1.34	6 (5.3%)	15.81±11.47	6 (7.0%)		
MO (10^3/μL)	Lower RI	0.00	1 (0.7%)	0.00±0.00	5 (2.7%)	-	0 (0.0%)	-	0 (0.0%)		
	Within RI	0.85±0.50	141 (97.2%)	0.71±0.55	175 (94.6%)	0.85±0.50	110 (96.5%)	0.84±0.49	81 (94.2%)	0.162	0.234
	Higher RI	3.26±1.61	3 (2.1%)	2.42±0.38	5 (2.7%)	2.47±0.24	4 (3.5%)	3.46±1.99	5 (5.8%)		

RI, reference intervals; SD, standard deviation; No., number; Pe, Pearson's chi-square test; Li, Linear by linear association; - means not calculable.

Table 12. Average of hematology and serum biochemistry according to the fecal score of 530 Korean indigenous calves (Continued)

Parame-ters	RI groups	Fecal scores								P value	
		0 (n=145)		1 (n=185)		2 (n=114)		3 (n=86)			
		Mean±SD	No. of calves	Mean±SD	No. of calves	Mean±SD	No. of calves	Mean±SD	No. of calves	Pe	Li
Alb (g/dL)	Lower RI	300.00±0.00	4 (2.8%)	237.04±74.15	27 (14.6%)	175.00±125.83	4 (3.5%)	260.00±54.77	5 (5.8%)	<0.001	<0.001
	Within RI	545.99±90.76	137 (94.5%)	551.79±107.37	112 (60.5%)	580.36±115.08	56 (49.1%)	608.82±79.27	34 (39.5%)		
	Higher RI	800.00±0.00	4 (2.8%)	882.61±87.70	46 (24.9%)	975.93±234.67	54 (47.4%)	1000.00±225.54	47 (54.7%)		
BUN* (mg/dL)	Lower RI	0.00±0.00	3 (2.1%)	0	1 (0.5%)	0	1 (0.9%)	0	1 (1.2%)	0.045	0.071
	Within RI	160.58±110.51	137 (94.5%)	233.04±114.45	157 (84.9%)	227.37±117.62	98 (86.0%)	232.95.117.13	76 (88.4%)		
	Higher RI	578.85±64.70	5 (3.4%)	598.21±47.07	27 (14.6%)	589.00±35.38	15 (13.2%)	587.63±31.17	9 (10.5%)		
GLU* (mg/dL)	Lower RI	-	0 (0.0%)	-	0 (0.0%)	-	0 (0.0%)	-	0 (0.0%)	<0.001	<0.001
	Within RI	5.45±5.93	143 (98.6%)	5.58±4.80	178 (96.2%)	12.27±7.38	92 (80.7%)	11.92±5.87	36 (41.9%)		
	Higher RI	30.00±2.82	2 (1.4%)	25.49±11.78	7 (3.8%)	29.36±4.40	22 (19.3%)	45.06±20.41	50 (58.1%)		
Fib* (mg/dL)	Lower RI	300.00±0.00	4 (2.8%)	237.04±74.15	27 (14.6%)	175.00±125.83	4 (3.5%)	260.00±54.77	5 (5.8%)	<0.001	<0.001
	Within RI	545.99±90.76	137 (94.5%)	551.79±107.37	112 (60.5%)	580.36±115.08	56 (49.1%)	608.82±79.27	34 (39.5%)		
	Higher RI	800.00±0.00	4 (2.8%)	882.61±87.70	46 (24.9%)	975.93±234.67	54 (47.4%)	1000.00±225.54	47 (54.7%)		
SAA (mg/L)	Lower RI	0.00±0.00	3 (2.1%)	0	1 (0.5%)	0	1 (0.9%)	0	1 (1.2%)	0.045	0.071
	Within RI	160.58±110.51	137 (94.5%)	233.04±114.45	157 (84.9%)	227.37±117.62	98 (86.0%)	232.95.117.13	76 (88.4%)		
	Higher RI	578.85±64.70	5 (3.4%)	598.21±47.07	27 (14.6%)	589.00±35.38	15 (13.2%)	587.63±31.17	9 (10.5%)		
Hp* (mg/dL)	Lower RI	-	0 (0.0%)	-	0 (0.0%)	-	0 (0.0%)	-	0 (0.0%)	<0.001	<0.001
	Within RI	5.45±5.93	143 (98.6%)	5.58±4.80	178 (96.2%)	12.27±7.38	92 (80.7%)	11.92±5.87	36 (41.9%)		
	Higher RI	30.00±2.82	2 (1.4%)	25.49±11.78	7 (3.8%)	29.36±4.40	22 (19.3%)	45.06±20.41	50 (58.1%)		

RI, reference intervals; SD, standard deviation; No., number; Pe, Pearson's chi-square test; Li, Linear by linear association; - means not calculable; *means significantly different in linear by linear association and Pearson's chi-square test.

Table 12. Average of hematology and serum biochemistry according to the fecal score of 530 Korean indigenous calves (Continued)

Parame-ters	RI groups	Fecal scores								P value	
		0 (n=145)		1 (n=185)		2 (n=114)		3 (n=86)			
		Mean±SD	No. of calves	Mean±SD	No. of calves	Mean±SD	No. of calves	Mean±SD	No. of calves	Pe	Li
Na* (mmol/L)	Lower RI	129.40±2.61	5 (3.4%)	125.10±6.38	10 (5.4%)	121.85±7.39	13 (11.4%)	123.22±6.40	23 (26.7%)	<0.001	<0.001
	Within RI	139.31±3.01	137 (94.5%)	139.22±3.34	172 (93.0%)	138.27±3.47	96 (84.2%)	137.95±3.8	63 (73.3%)		
	Higher RI	152.67±5.03	3 (2.1%)	149.33±1.53	3 (1.6%)	150.00±1.87	5 (4.4%)	-	0 (0.0%)		
K* (mmol/L)	Lower RI	4.30	1 (0.7%)	4.10±0.10	3 (1.6%)	3.80	1 (0.9%)	2.80	1 (1.2%)	<0.001	<0.001
	Within RI	5.60±0.57	139 (95.9%)	5.67±0.51	174 (94.1%)	5.75±0.60	95 (83.3%)	5.71±0.58	66 (76.6%)		
	Higher RI	7.40±0.34	5 (3.4%)	7.63±0.35	8 (4.3%)	8.72±1.20	18 (15.8%)	9.50±0.91	19 (22.1%)		
Cl (mmol/L)	Lower RI	88.67±4.16	3 (2.1%)	89.50±3.54	2 (1.1%)	88.29±2.87	7 (6.1%)	88.92±3.26	12 (14.0%)	<0.001	0.476
	Within RI	99.77±2.57	136 (93.8%)	99.40±2.38	181 (97.8%)	99.24±2.49	107 (93.9%)	99.08±0.31	74 (86.0%)		
	Higher RI	108.50±2.35	6 (4.1%)	108.00±1.41	2 (1.1%)	-	0 (0.0%)	-	0 (0.0%)		

RI, reference intervals; SD, standard deviation; No., number; Pe, Pearson's chi-square test; Li, Linear by linear association; [a]-means not calculable; *means significantly different in linear by linear association and Pearson's chi-square test.

Table 13. The results of receiver operating characteristic curve analysis, sensitivity-specificity analysis, and determination of cut-off values for fecal score 3 in Korean indigenous calves

Blood parameters	AUC	SE	95% Confidence Intervals	P values	Cut points	Sensitivities	Specificities
BUN (mg/dL)*	0.623	0.035	0.554~0.692	<0.001	9.5	0.600	0.561
GLU (mg/dL)	0.271	0.032	0.207~0.335	<0.001	83.5	0.302	0.306
Na (mmol/L)	0.366	0.026	0.259~0.392	<0.001	137.5	0.398	0.371
K (mmol/L)*	0.599	0.037	0.526~0.672	<0.001	5.75	0.583	0.542
Fib (mg/dL)*	0.706	0.032	0.644~0.768	<0.001	650.0	0.686	0.619
Hp (mg/dL)*	0.847	0.023	0.801~0.893	<0.001	12.50	0.744	0.759

AUC, area under the ROC curve; ROC, receiver operating characteristic; SE, standard error; *mean AUC > 0.5.

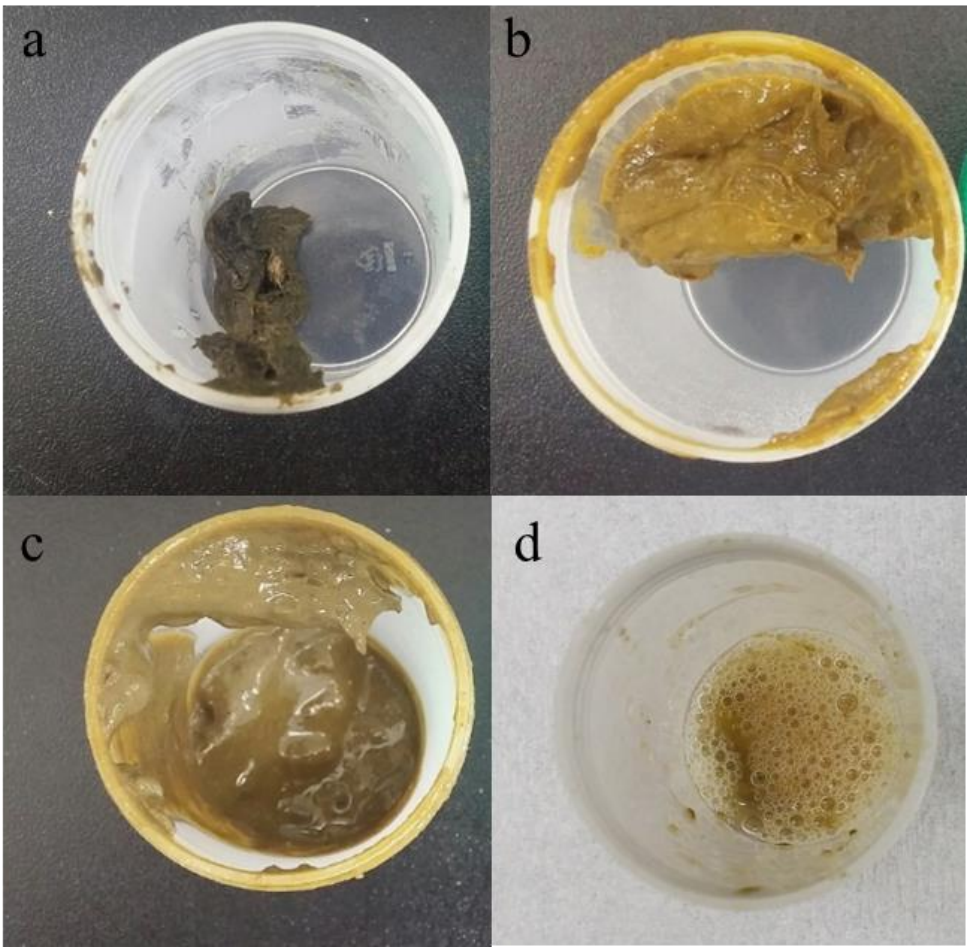


Fig. 4. Collected feces description according to the fecal scores. (a) fecal score 0 (normal), (b) fecal score 1 (semi-formed), (c) fecal score 2 (loose but bedding), (d) fecal score 3 (watery). The fecal consistency scoring system is a 4-level scoring system based on the degree of fluidity of the feces created by the University of Wisconsin-Madison School of Veterinary Medicine (McGuirk, 2008).

Chapter II

The Prevalence of Infectious Pathogens of Calf Diarrhea in Korean Indigenous Calves

Abstract

Infectious calf diarrhea is one of the most significant diseases of neonatal calves. A total of 544 feces from KIC in 2016~2017 were obtained to investigate selected seven pathogens causing calf diarrhea: bovine rotavirus (BRV), bovine coronavirus (BCV), *Cryptosporidium parvum*, bovine viral diarrhea virus (BVDV), *Eimeria* species, *Escherichia coli* K99, and *Salmonella* species. Among 544 feces collected in 2016~2017, the number of feces that scored 0, 1, 2, and 3 were 153, 187, 116, and 88, respectively. In this study, fecal scores were significantly higher in summer and pathogens-involved feces ($p < 0.05$) and the number of detected pathogens ($p < 0.01$). Among 7 pathogens, the detection rates and mean fecal scores

for each were as follows: *Eimeria* spp. (27.4%, 1.36), BRV (8.8%, 1.85), BCV (8.5%, 1.36), *C. parvum* (4.4%, 1.91), BVDV (0.7%, 0.5), and *E. coli* K99 (0.2%, 3). *Salmonella* spp. was not detected in any of the 544 fecal samples from KIC. The detection rates of BRV ($p < 0.001$) and *C. parvum* ($p < 0.01$) were getting increased as fecal scores were increased. Moreover, BCV showed a significant association of concurrent infection with *C. parvum* ($p < 0.01$) and bovine rotavirus ($p < 0.05$). These results will be fundamental to understanding the host–agent ecology and dynamics of the pathogens in diarrhea in KIC and to developing effective prevention strategies including vaccine development.

Keywords: calf diarrhea, Korean indigenous calves, prevalence, infectious pathogens

2.1. Introduction

Infectious calf diarrhea is one of the most significant diseases of neonatal calves. It has affected the morbidity and mortality of neonatal calves and their growth performances and has caused worldwide economic loss (Cho and Yoon, 2014). Calf diarrhea is caused by a number of factors including pathogens, environmental factors, management factors and the status of the animals (Klein-Jöbstl *et al*., 2014). If calf diarrhea is occurred, calves will lose their body fluids which leads to electrolyte imbalance, metabolic changes, plasma ion concentration changes, acidosis, and sometimes death. For the calf owner, calf diarrhea can cause extensive economic losses due to the need for treatments, diagnostics, manual labor, and veterinary intervention. Even though various methods have been designed to treat calf diarrhea, prevention is still the best approach to reduce the disease, and monitoring for infectious agents is one of the most important preventive actions (Pereira *et al*., 2017).

Many researchers and reports worldwide have attempted to determine the prevalence of infectious pathogens in calf diarrhea (Izzo *et al*., 2011; Peter *et al*., 2016; Smith, 2012; Uhde *et al*., 2008). Major pathogens causing calf diarrhea in these reports were viruses (BCV, BRV, BVDV), bacteria (*E. coli* K99 and *Salmonella* spp.), and protozoa (*C. parvum* and *Eimeria* spp.). Some of the agents are known to be detected not only in diarrheic feces but also in normal feces.

In the ROK, like other countries, calf diarrhea has had a serious impact on calf death. According to previous studies, 68.7% of calf deaths in KIC and 53.4% in dairy calves were caused by digestive diseases (Hur *et al*., 2013; Kim *et al*., 2015). Additionally, there have been several recent reports investigating pathogens that cause calf diarrhea in the ROK (Kim *et al*., 2018; Lee *et al*., 2016; Park *et al*., 2018). However, most of them have been focused on specific pathogens from calf feces. As calf diarrhea can be caused by a variety of pathogens, it is necessary to simultaneously analyze different kinds of pathogens. This study was performed to investigate the distribution of infectious pathogens of calf diarrhea in KIC aged less than 60 days in various regions of the ROK and to discern their association with diarrhea.

2.2. Materials and Methods

Ethical statement

All procedures were performed according to ethical guidelines for the use of animal samples, as approved by the Institutional Animal Care and Use Committee (IACUC) in Jeonbuk National University (IACUC No. JBU 2016-00026).

Sample collection

In this study, calves up to 60 days of age in Korean indigenous cattle farms in different areas of the ROK were selected for feces collection in 2016~2017. Feces were obtained by digital rectal palpation of the calves. All feces were scored as 0 to 3 according to fecal consistency (McGuirk, 2008) and stored in 50 ml specimen bottles (SPL Life Sciences, Pocheon, the ROK) at 4°C until they were transported to the laboratory.

Laboratory procedures

Each feces were treated differently depending on the target agent, according to previously reported methods (Cho *et al*., 2010; Kim *et al*., 2018). To detect the 6 infectious agents causing calf diarrhea (BCV, BRV, BVDV, *C. parvum*, *E. coli* K99, *Salmonella* spp.), feces were suspended in 0.01 M phosphate-buffered saline to make 30% fecal homogenates and centrifuged for 1 min at 100 × g. A supernatant was used to extract the total nucleic acid using MagMAX^{TM} Total Nucleic Acid Isolation Kit

(Thermo Fisher Scientific, Waltham, MA). All procedures were done according to the manufacturer's instructions. All extracts were stored at -70°C until real-time polymerase chain reaction (PCR) was performed. Real-time PCR was performed with the Path-IDTM Multiplex One-Step RT-PCR kit (Life technologies, Carlsbad, CA) according to the manufacturer's recommended protocols in a 25 ul reaction volume using 8 ul of extracted template and 17 ul of the reaction mixture. Two types of real-time PCR were performed using specific primer sets for each pathogen in Table 14: one for the 3 viruses (BCV, BRV, BVDV) and the other for the bacteria and protozoa (*C. parvum*, *E. coli* K99, *Salmonella* spp.). Equal volumes of primers and probes were mixed for each target agent and the final concentration of each primer and probe was 0.2 uM. Real-time PCR was performed using ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Cycling conditions were as follows: (a) reverse transcription for 10 min at 45°C (omitted for bacteria/protozoa set PCR); (b) a 10 min activation step at 95°C; and (c) 40 cycles of 15 sec at 95°C and 60 sec at 60°C. After a 40 cycles reaction, samples with cycle threshold value less than 35 for targets were considered positive.

To detect *Eimeria* spp., all feces were suspended in a solution of 2.5% potassium dichromate and then transported to the laboratory. In the laboratory, feces were analyzed to detect oocysts using the floatation methods with Sheather's solution (saturated sugar solution; specific gravity 1.28) and examined microscopically (×400 magnification) based on the morphological features of the oocysts of the *Eimeria* spp.

Statistical analysis

All statistical methods were performed by SPSS (Version 25.0; IBM Corp., Armonk, NY). The PCR results for each pathogen were recorded as positive or negative and categorized based on fecal scores. The association between fecal scores, the presence of pathogens, and each pathogen were compared using the Pearson's $\chi 2$ and Fischer's exact test. Pearson's $\chi 2$ and linear by linear association were analyzed to evaluate the association between fecal scores, each pathogen, and the number of concurrent infected pathogens. The probability of concurrent detection among pathogens was analyzed using a linear regression model. Odds ratios with 95% confidence intervals were calculated to assess the likelihood of association.

2.3. Results

Sample collection

To investigate the prevalence of pathogens regarding calf diarrhea, 544 feces were collected from KIC on 10 Korean indigenous cattle farms in 2016~2017. Ten farms were described in Table 9 in Chapter 1. Among 544 feces in 2016~2017, the number of feces scored 0, 1, 2 and 3 were 153, 187, 116 and 88, respectively.

The association between fecal scores and seasons

To identify the association between fecal scores and seasons in KIC, 544 feces in 2016~2017 were described by seasons and fecal scores (Table 15, A1). Of 544 feces in total, 282 feces (fecal score 0: 83 feces, fecal score 1: 110 feces, fecal score 2: 59 feces, fecal score 3: 30 feces), 132 feces (fecal score 0: 25 feces, fecal score 1: 41 feces, fecal score 2: 28 feces, fecal score 3: 38 feces), and 130 feces (fecal score 0: 45 feces, fecal score 1: 36 feces, fecal score 2: 29 feces, fecal score 3: 20 feces) were collected in spring (March to May), summer (June to August), and autumn (September to November), respectively. Sampling was not conducted in winter. Fecal scores in summer were significantly higher than other seasons ($p = 0.001$).

The association between fecal scores and pathogens

To identify the association between fecal scores and pathogens in KIC, 544

feces in 2016~2017 were described by farms, pathogen presence and fecal scores (Table 16, A2). The feces that more than one pathogen was detected were considered pathogens positive. Of 544 feces in total, pathogens were not detected in 314 feces (fecal score 0: 96 feces, fecal score 1: 117 feces, fecal score 2: 62 feces, fecal score 3: 39 feces). On the other hand, pathogen was detected in 230 feces (fecal score 0: 57 feces, fecal score 1: 70 feces, fecal score 2: 54 feces, fecal score 3: 49 feces). Fecal scores with pathogens were significantly higher than those without pathogens ($p = 0.003$).

To identify the association between fecal scores and each pathogen in KIC, each pathogen from 544 feces in 2016~2017 were described according to fecal scores (Table 17). As a result, *Eimeria* spp. (27.4%, 149/544) was the most prevalent pathogen, followed by BRV (8.8%, 48/544), BCV (8.5%, 46/544), *C. parvum* (4.4%, 24/544), BVDV (0.7%, 4/544), and *E. coli* K99 (0.2%, 1/544). There was no *Salmonella* spp. in 544 feces in KIC. Among 7 pathogens, the detection rates of BRV ($p < 0.001$) and *C. parvum* ($p < 0.01$) were getting increased as fecal scores were increased.

To identify the prevalence of each pathogen in each farm, each pathogen from 544 feces in 2016~2017 were described according to the farms (Table 18). More than one pathogens were detected in all 10 farms. As a result, *Eimeria* spp., BRV, BCV, *C. parvum*, BVDV, and *E. coli* K99 was detected in 8 farms, 9 farms, 8 farms, 6 farms, 3 farms, and 1 farm, respectively. There was no *Salmonella* spp. in 544 feces in KIC.

The concurrent infected pathogens and their associations

Of 544 feces, 230 feces had more than one pathogens (Table 19). In details, 196 feces had 1 pathogen, 28 feces had 2 different pathogens, 4 feces had 3 different pathogens, and 2 feces had 4 different pathogens. As the number of concurrent infected pathogens increased, the fecal score also increased significantly ($p < 0.01$). The number of concurrent infected pathogens in 544 feces of KIC were compared according to farms (Table 20). Among 10 farms, more than 2 pathogens were detected in 5 farms. Concurrent infected pathogens were described in Table 21 and their probability of detecting pathogens together in 5 farms (Farm 2, 3, 4, 8, and 9) where more than 2 pathogens were detected was described in Table 22. Among 7 pathogens, BCV presence was significantly correlated with *C. parvum* ($p < 0.01$) and BRV ($p < 0.05$).

2.4. Discussion

In this study, the prevalence of the 7 pathogens in the feces of 544 KIC and the association with the pathogens causing calf diarrhea were demonstrated. The relationship between seasons, pathogen presence, and fecal scores were analyzed. In this research, the average of fecal scores in summer was significantly higher than spring or autumn ($p = 0.001$). However, the pathogen was not detected highest in summer (40.9%). This result might be caused by environmental factors including stress by high temperature and high humidity in the rainy season. In this research, sampling was not conducted in winter, because the birth rate of calves in 10 farms was too low to conduct sampling. The fecal scores of feces in the presence of pathogens were significantly higher than no pathogen, which was consistent with the findings from other countries (Cho *et al*., 2013). Even though there are many factors that affect calf diarrhea, which were not investigated in this study, it is clear that infectious agents are one of the primary factors related to diarrhea in KIC given the correlation between fecal consistency and presence of pathogens.

Eimeria spp. was detected in 27.4% of the 544 KIC, making it the most prevalent pathogen of the 7 examined in this research. This detection rate is similar to that in other reports from the ROK (Kim *et al*., 2018). The detection rates of *Eimeria* spp. was not significantly different according to fecal scores. However, because *Eimeria* spp. has been shown to be found frequently in the feces of healthy

calves (Gulliksen *et al*., 2009), this result was conceivable. The amount of oocyte secretion was not investigated in this research, but the excretion of oocytes of *Eimeria* spp. is known to be strongly correlated with diarrhea, and thus, further research should investigate the correlation between diarrhea in KIC and the amount of *Eimeria* spp. excreted.

The detection rates of BRV was significantly getting higher as fecal score increased ($p < 0.001$). These results demonstrate that rotavirus is an important infectious agent that can negatively affect the health of calves; this finding is consistent with that of earlier reports (Bartels *et al*., 2010; Cho *et al*., 2013). Diarrhea associated with BRV infection was discovered despite the presence of a commercial vaccine for BRV. Therefore, continued surveillance and characterization of BRV and ongoing evaluation of the vaccine against BRV strains in the field are necessary to control BRV-associated diarrhea in KIC.

The detection rates of BCV was not significantly different according to fecal scores. Even though BCV is known as one of the main pathogens associated with calf diarrhea, BCV was also detected in normal feces in previous reports (Izzo *et al*., 2011; Kirisawa *et al*., 2007). According to the results of this study, BRV affected calf diarrhea more than BCV in KIC. This finding was similar to that of some reports wherein BRV was detected more than BCV in the diarrhea samples of calves in Algeria (Akam *et al*., 2011), Australia (Izzo *et al*., 2011), and India (Rai *et al*., 2011). However, some other reports have shown that BCV was detected more often than BRV in Ethiopia (Abraham *et al*., 1992) and Costa Rica (Pérez *et al*.,

1998). This difference might be related to different diagnostic methods, farm management practices in different regions, hygiene status, geographical locations, or other factors (Ammar *et al*., 2014).

The detection rates of *C. parvum* was significantly getting higher as fecal score increased ($p < 0.01$). There have been many reports emphasizing the effects of *C. parvum* infection in calf diarrhea in other countries (Cho *et al*., 2013, Izzo *et al*., 2011, Trotz-Williams *et al*., 2005). Because there is no worldwide commercial vaccine for *C. parvum*, maintaining good herd sanitation and keeping sick calves away from healthy calves are important in preventing *C. parvum* infections.

Bovine viral diarrhea virus was detected in only 4 calves. The detection rate of BVDV in this study was less than that in previous research (Han *et al*., 2018). The reason might be related to the type of sample. According to a previous report, ear notch, skin fold biopsies, and nasal swabs showed reliable results for the detection of BVDV; however, rectal swab was not as reliable in calves. Because feces were used to detect BVDV in this research, the results could be inaccurate in assessing the actual prevalence of BVDV infection in KIC.

There was only one calf positive for *E. coli* K99 in this research. This result was consistent with that of other reports in the ROK that no *E. coli* strain expressing K99 was detected in isolated samples from cattle farms (Shin *et al*., 2014). Considering that commercial vaccines against K99 containing *E. coli* were mainly used, these commercial vaccines may have been good effects on the prevalence of *E. coli* K99. However, many other *E. coli* containing other virulence factors, such as

adhesins (F17, F41, Intimin, CS31A, Afimbrial adhesion) and toxins (heat-stable enterotoxin, shiga toxin1, shiga toxin2) were detected in the ROK (Hur *et al*., 2013). For the investigation of calf diarrhea affected by *E. coli*, additional investigation of *E. coli* virulence factors should be needed.

Salmonella spp. occurring calf diarrhea was not detected in this research. However, since *Salmonella* infection in other livestock and human have been reported in the ROK (Kang *et al*., 2017; Oh *et al*., 2016), it is necessary to conduct ongoing monitoring of *Salmonella* infection in KIC.

In this study, the fecal scores significantly increased ($p < 0.01$) as the number of concurrently infected pathogens increased, and BCV were significantly associated with *C. parvum* and BRV, although BCV itself did not show any significant difference based on the fecal scores. Some studies have reported that viral infections may be a predisposing factor of *C. parvum* infection in pigs and humans (Núñez *et al*., 2003; Putignani and Menichella, 2010). As no effective treatment exists for *C. parvum*, the control of calf diarrhea caused by *C. parvum* should rely on effective management of farms and the prevention of viral infections related to *C. parvum*.

2.5. Conclusions

In this study, the detection rates and mean fecal scores for each were as follows: *Eimeria* spp. (27.4%, 1.36), BRV (8.8%, 1.85), BCV (8.5%, 1.36), *C. parvum* (4.4%, 1.91), BVDV (0.7%, 0.5), and *E. coli* K99 (0.2%, 3). *Salmonella* spp. was not detected in any of the 544 fecal samples from KIC. Among 7 pathogens, *C. parvum* and BRV appeared to be the primary pathogens that significantly influence calf diarrhea in KIC and BCV showed significant association with BRV and *C. parvum*. The findings of this study highlighted the importance of viral pathogens in calf diarrhea in the ROK. This information may help better understanding of calf diarrhea in KIC and the development of strategies for preventing calf diarrhea in the ROK.

Table 14. Nucleotide sequences of multiplex real-time PCR primers for infectious agents causing calf diarrhea

Target sets	Microbial agents	PCR primers, probes	5'- nucleotide sequences - 3'	References
Viruses	Bovine viral diarrhea virus	Forward	GGG NAG TCG TCA RTG GTT CG	Mahlum *et al*., 2002
		Reverse	GTG CCA TGT ACA GCA GAG WTT TT	
		Probe (CY5/BHQ2)	CCA YGT GGA CGA GGG CAY GC	
	Bovine coronavirus	Forward	CTA GTA ACC AGG CTG ATG TCA ATA CC	Cho *et al*., 2010
		Reverse	GGC GGA AAC CTA GTC GGA ATA	
		Probe (FAM/MGB)	CGC CTG ACA TTC TCG ATC	
	Bovine rotavirus	Forward	TCA ACA TGG ATG TCC TGT ATT CCT	Cho *et al*., 2010
		Reverse	TCC CCC AGT TTG GAA TTC ATT	
		Probe (VIC/MGB)	TCA AAA ACT CTT AAA GAT GCA AG	
Bacteria/Parasites	*Escherichia coli* K99	Forward	GCT ATT AGT GGT CAT GGC ACT GTA G	West *et al*., 2007
		Reverse	TTT GTT TTC GCT AGG CAG TCA TTA	
		Probe (FAM/BHQ1)	ATT TTA AAC TAA AAC CAG CGC CCG GCA	
	Cryptosporidium parvum	Forward	CAA ATT GAT ACC GTT TGT CCT TCT GT	Guy *et al*., 2003
		Reverse	GGC ATG TCG ATT CTA ATT CAG CT	
		Probe (JOE/BHQ1)	TGC CAT ACA TTG TTG TCC TGA CAA ATT GAA	
	Salmonella species	Forward	GGG NAG TCG TCA RTG GTT CG	Moore *et al*., 2007
		Reverse	GTG CCA TGT ACA GCA GAG WTT TT	
		Probe (CY5/BHQ2)	CCA YGT GGA CGA GGG CAY GC	

PCR, polymerase chain reaction.

Table 15. Fecal scores and seasons description of feces from 544 Korean indigenous calves (KIC) in 10 farms

Seasons	Fecal scores (No. of KIC, %)			
	0	**1**	**2**	**3**
Spring (n=282)	83 (29.4)	110 (39.0)	59 (20.9)	30 (10.6)
Summer (n=132)	25 (18.9)	41 (31.1)	28 (21.2)	38 (28.8)
Autumn (n=130)	45 (34.6)	36 (27.7)	29 (22.3)	20 (15.4)
Total (n=544)	**153 (28.1)**	**187 (34.4)**	**116 (21.3)**	**88 (16.2)**

Fecal scores in total were significantly different by seasons ($p = 0.001$).

Table 16. Fecal scores and pathogens presence of feces from 544 Korean indigenous calves (KIC) in 10 farms

Pathogen presence	Fecal scores (No. of KIC, %)			
	0	**1**	**2**	**3**
Negative (n=314)	96 (30.6)	117 (37.3)	62 (19.7)	39 (12.4)
Positive (n=230)	57 (24.8)	70 (30.4)	54 (23.5)	49 (21.3)
Total (n=544)	**153 (28.1)**	**187 (34.4)**	**116 (21.3)**	**88 (16.2)**

[a]Fecal scores in total were significantly different by pathogen presence (p = 0.003).

Table 17. The detection rates of 7 pathogens in feces from 544 Korean indigenous calves (KIC) according to fecal scores

Pathogens	Fecal scores (No. of KIC, %)					P value	
	0 (n= 153)	1 (n=187)	2 (n=116)	3 (n=88)	Total (n=544)	Pe	Li
Eimeria spp.	42 (27.5%)	43 (23.0%)	32 (27.6%)	32 (36.4%)	149 (27.4%)	0.146	0.140
BRV	4 (2.6%)	13 (7.0%)	17 (14.7%)	14 (15.9%)	48 (8.8%)	<0.001	<0.001
BCV	13 (8.5%)	12 (6.4%)	12 (10.3%)	9 (10.2%)	46 (8.5%)	0.594	0.436
C. parvum	3 (2.0%)	4 (2.1%)	9 (7.8%)	8 (9.1%)	24 (4.4%)	0.007	0.001
BVDV	2 (1.3%)	2 (1.1%)	0 (0.0%)	0 (0.0%)	4 (0.7%)	0.478	0.144
E. coli K99	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (1.1%)	1 (0.2%)	0.158	0.093
Salmonella spp.	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	-	-

Pe, Pearson's chi-square test; Li, linear by linear association; BRV, bovine rotavirus; BCV, bovine coronavirus; *C. parvum, Cryptosporidium parvum*; BVDV, bovine viral diarrhea virus; *E. coli*, *Escherichia coli* K99; -, not calculable.

Table 18. The prevalence of 7 pathogens in feces from 544 Korean indigenous calves (KIC) according to the farms

Pathogens	Farm IDs (No. of KIC)											No. of farms positive for pathogens (%)
	1 (n=19)	2 (n=109)	3 (n=175)	4 (n=29)	5 (n=9)	6 (n=36)	7 (n=8)	8 (n=85)	9 (n=61)	10 (n=13)	Total (n=544)	
Eimeria spp.	4	43	42	17	0	2	0	17	22	2	149	8 (80)
BRV	1	11	16	3	0	3	1	7	5	1	48	9 (90)
BCV	2	11	14	3	1	2	0	5	8	0	46	8 (80)
C. parvum	0	3	9	4	0	0	0	1	5	2	24	6 (60)
E. coli K99	0	0	0	1	0	0	0	0	0	0	1	1 (10)
BVDV	0	0	1	0	0	0	0	1	2	0	4	3 (30)
Salmonella spp.	0	0	0	0	0	0	0	0	0	0	0	0 (0)

No., Number; BRV, bovine rotavirus; BCV, bovine coronavirus; *C. parvum*, *Cryptosporidium parvum.*

Table 19. The comparison of concurrent infection in feces from 544 Korean indigenous calves according to fecal scores

No. of Pathogens	Fecal scores (No. of KIC, %)					P value	
	0 (n= 153)	1 (n=187)	2 (n=116)	3 (n=88)	Total (n=544)	Pe	Li
0	96 (62.7)	117 (62.6)	62 (53.4)	39 (44.3)	314 (57.7)		
1	51 (33.3)	66 (35.3)	40 (34.5)	39 (44.3)	196 (36.0)		
2	5 (3.3)	4 (2.1)	12 (10.3)	7 (8.0)	28 (5.1)	0.001	<0.001
3	1 (0.7)	0 (0.0)	2 (1.7)	1 (1.1)	4 (0.7)		
4	0 (0.0)	0 (0.0)	0 (0.0)	2 (2.3)	2 (0.4)		

Pe, Pearson's chi-square test; Li, linear by linear association.

Table 20. The comparison of concurrent infection in feces from 544 Korean Indigenous calves (KIC) according to farms

Pathogens	Farm IDs (No. of KIC)											No. of farms positive for pathogens (%)
	1 (n=19)	2 (n=109)	3 (n=175)	4 (n=29)	5 (n=9)	6 (n=36)	7 (n=8)	8 (n=85)	9 (n=61)	10 (n=13)	Total (n=544)	
0	12	53	101	9	8	29	7	55	32	8	314	10 (100)
1	7	47	66	13	1	7	1	29	20	5	196	10 (100)
2	0	7	8	6	0	0	0	1	6	0	28	5 (50)
3	0	1	0	1	0	0	0	0	2	0	4	3 (30)
4	0	1	0	0	0	0	0	0	1	0	2	2 (20)

No., Number.

Table 21. The prevalence of concurrent infected pathogens in feces from 544 Korean indigenous calves (KIC)

The number of pathogens concurrently infected	Infectious pathogens	Number of KIC
0 (No infection)	No pathogens found	**314**
1 (Single infection)	*Eimeria* spp.	121
	BRV	32
	BCV	28
	C. parvum	13
	BVDV	2
	E. coli K99	0
	Subtotal	**196**
2 (Double infection)	BRV + *Eimeria* spp.	8
	BCV + *Eimeria* spp	7
	C. parvum + *Eimeria* spp.	5
	BCV + *C. parvum*	3
	Eimeria spp. + *E. coli* K99	1
	Eimeria spp. + BVDV	2
	BCV+ BRV	2
	Subtotal	**28**
3 (Triple infection)	BCV+BRV+ *Eimeria* spp.	1
	BCV+BRV+ *C. parvum*	2
	BCV+*C. parvum*+ *Eimeria* spp.	1
	Subtotal	**4**
4 (Quadruple infection)	BCV+BRV+ *C. parvum* + *Eimeria* spp.	2
	Subtotal	**2**
	Total	**544**

BRV, bovine rotavirus; BCV, bovine coronavirus; *C. parvum*, *Cryptosporidium parvum.*

Table 22. The association strength of concurrent detected pathogens in 544 Korean indigenous calves

Reference pathogens	Associated pathogens	P value	Odds ratio (95% CI)
C. parvum	BCV	0.004	4.31 (1.59~11.71)
BCV	BRV	0.020	2.74 (1.17~6.40)
	C. parvum	0.004	4.31 (1.59~11.71)
BRV	BCV	0.020	2.74 (1.17~6.40)

BRV, bovine rotavirus; BCV, bovine coronavirus; *C. parvum*, *Cryptosporidium parvum*; CI, confidence interval

Chapter III

Detection of Ten Viruses Associated with Calf Diarrhea by Next-Generation Sequencing

Abstract

Calf diarrhea is a major concern in the cattle industry worldwide, resulting in significant economic losses and one of the major causes of this disease has been infectious pathogens. In this study, the prevalence of 7 pathogens (BRV, BCV, BVDV types 1 and 2, *Cryptosporidium parvum*, *Giardia* spp., and *Eimeria* spp.) associated with calf diarrhea were investigated by polymerase chain reaction (PCR) and metagenomic approach targeting viral pathogens was applied to other pathogens causing calf diarrhea in the ROK. In total, 810 feces from KIC were collected, composed of 526 normal feces (comprising 267 feces with a fecal score of 0 and 259 feces with a fecal score of 1) and 284 diarrheic feces (comprising 178 feces with a fecal score of 2 and 106 feces with a fecal score of 3). All 7 pathogens were detected

by PCR in feces and their detection rates and mean fecal scores for each were as follows: BRV (14.0%, 1.41), BCV (3.2%, 1.42), BVDV1 (2.1%, 1.35), BVDV2 (4.9%, 1.33), *C. parvum* (9.8%, 1.66), and *Eimeria* spp. (1.9%, 1.73), and *Giardia* spp. (0.9%, 0.71). Among 7 pathogens, BRV ($p = 0.004$), *C. parvum* ($p < 0.001$), and *Eimeria* spp. ($p = 0.027$) increased as fecal scores increased. Twenty one feces that were negative for all pathogens tested in this study were subjected to high-throughput sequencing to identify viral pathogens associated with calf diarrhea. As a result, the nearly complete genomic sequences of bovine astrovirus (BAstV), bovine enterovirus (BEV), bovine kobuvirus (BKoV), bovine nebovirus (BNeV), bovine norovirus (BNoV), bovine boosepivirus B (BooV), bovine parechovirus (BParV), bovine torovirus (BToV), *C. parvum* virus 1 (CSpV1), and hunnivirus were identified. This study represents the first investigation to identify the presence of BooV, CSpV1, and hunnivirus and it provides a comprehensive description of the nearly complete genomes of ten novel viruses associated with calf diarrhea in the ROK. The findings of this study would contribute to a better understanding of the epidemiology and molecular characteristics of calf diarrhea-associated pathogens in the ROK.

Keywords: calf diarrhea, infectious pathogens, next generation sequencing, novel viruses

3.1. Introduction

Calf diarrhea is a major concern in the cattle industry, resulting in significant economic losses due to decreased growth and increased morbidity and mortality in neonatal calves (Cho and Yoon, 2014). Calf diarrhea is caused by various factors, including environmental factors, management, and infectious causes (Meganck *et al*., 2015). Among these factors, infectious causes were considered one of the primary factors of calf diarrhea since the presence of pathogens is significantly related to the presence of calf diarrhea (Cho *et al*., 2013). Historically, major pathogens, including BRV, BCV, BVDV, *C. parvum*, *Giardia* spp., and *Eimeria* spp. have been associated with calf diarrhea (Singh *et al*., 2006; Foster *et al*., 2009; Lee *et al*., 2016; Chae *et al*., 2021).

The investigation of the causative agent of calf diarrhea has been reported by many researchers, even in the ROK (Lee *et al*., 2019; Ryu *et al*., 2020b; Chae *et al*., 2021; Park *et al*., 2023). However, most reports have used traditional diagnostic methods, especially PCR, to target specific pathogens. Traditional diagnostic methods for investigating infectious diseases, such as bacterial and viral cultures, serological assays, and microscopy, are limited by their inability to detect unculturable or unknown pathogens. This has resulted in a substantial number of undiagnosed cases and delays in appropriate treatment, particularly in cases where the etiological agent is a novel or emerging pathogen.

The metagenomic approach and next-generation sequencing (NGS) have

revolutionized the field of pathogen detection in recent years (Kubacki *et al*., 2021). The ability to sequence millions of DNA fragments in a single run has allowed for the detection of a vast range of microorganisms, including viruses, bacteria, fungi, and parasites, without the need for prior knowledge or culturing, enabling the identification of multiple pathogens in a single sample and providing a more comprehensive understanding of the etiology of the diseases. Using this technology, diagnosis and molecular characterization of previously undiagnosed causes would be possible (Belák *et al*., 2013; Qiu *et al*., 2022).

This study was performed to investigate the distribution of infectious pathogens (BRV, BCV, BVDV, *C. parvum*, *Giardia* spp., and *Eimeria* spp.) of calf diarrhea in calves in the ROK using PCR method and metagenomic approach targeting viral pathogens that had been overlooked due to their lack of recognition as major causative agents.

3.2. Materials and Methods

Sample collection

In this study, feces from KIC up to 60 days of age in 15 different farms in the ROK were submitted to the animal diagnostic laboratories of Animal Industry Data Korea, Ltd. for disease diagnosis. Feces were obtained by digital rectal palpation of the calves and scored as 0 to 3 according to fecal scoring system included in the calf health scoring guide created by the University of Wisconsin-Madison School of Veterinary Medicine by field veterinarians (McGuirk, 2008). Feces scored 2 and 3 were considered as diarrhea. All feces were stored in 50 ml specimen bottles (SPL Life Sciences, Pocheon, Korea) at 4°C until they were transported to the laboratory.

Pathogen detection by polymerase chain reaction

After feces were transported to the laboratory, feces were suspended in 0.01 M phosphate-buffered saline to make 30% fecal homogenates and centrifuged for 1 min at 100 × g. Total nucleic acids were extracted from the supernatant using the MagMAX^{TM} Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's instructions. All extracts were stored at – 70°C until pathogen detection was performed. Pathogen detection was performed using PCR or real-time PCR depending on targeted pathogen. Specific primer/probe sets

for each pathogen were described in Table 23 (Guy *et al*., 2003; Letellier *et al*., 2003; Solberg *et al*., 2009; Thanthrige-Don *et al*., 2018; Kim *et al*., 2022).

To detect BRV and BCV, RT - PCR was performed with the PrimeScript™ One-Step RT-PCR Kit Ver. 2 (Takara Bio Inc., Shiga, Japan) according to the manufacturer's recommended protocols in a 25 µl reaction volume using 2 µl of extracted template and 23 µl of the reaction mixture containing 1 µl of PrimeScript 1 step Enzyme Mix, 12.5 µl of 2 × 1-Step Buffer, 1 µl of the primer mixture, and 8.5 µl of RNase-Free dH_2O. Final concentration of primer was 0.4 µM. RT-PCR was performed at 50°C for 30 min, 94°C for 2 min, 35 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min, and final extension at 72°C for 5 min. All PCR products were separated by gel electrophoresis on 1.0% agarose gel stained with commercial nucleic acid staining solution (RedSafe™ nucleic acid staining solution, Intron Biotechnology Inc., Seongnam, Korea) and subjected to direct sequencing using a dideoxy termination with an automatic sequencer (3730xl capillary DNA Analyzer; Applied Biosystems, Foster City, CA).

Real-time PCR were performed using specific primer/probe sets for each pathogen (BVDV 1, BVDV2, *C. parvum*, *Eimeria* spp, *Giardia* spp.) (Table 23). Real-time PCR was performed with the GoTaq One-Step RT-qPCR System (Promega, Madison, WI) according to the manufacturer's recommended protocols in a 10 µl reaction volume using 2 µl of extracted template and 8 µl of the reaction mixture containing 5 µl of GoTaq qPCR Master Mix 2X, 0.2 µl of GoScript™ RT Mix for 1-Step RT-qPCR 50X, 2 µl of the primer and probe mixture, and 0.8 µl of

RNase-Free dH_2O. Final concentration of primer and probe was 0.3 μM and 0.2 μM, respectively. Real-time PCR was performed using CFX Opus 96 Real-Time PCR System (Applied Biosystems, Foster City, CA). Cycling conditions were as follows: (a) reverse transcription for 10 min at 45°C (omitted for *C. parvum*, *Eimeria* spp. and *Giardia* spp. PCR); (b) a 10 min activation step at 95°C; and (c) 40 cycles of 15 sec at 95°C and 60 sec at 60°C. After a 40 cycles reaction, samples with cycle threshold value less than 35 for targets were considered positive.

Pathogen detection by next generation sequencing

Total RNA concentration was calculated using Quant-IT RiboGreen (Invitrogen, Carlsbad, CA). To assess the integrity of total RNA, samples were run on a TapeStation RNA screentape (Agilent, Santa Clara, CA). A library was independently prepared with 0.5 ug of total RNA for each sample using the Illumina Stranded Total RNA Library Prep with Ribo-Zero Plus (Illumina, Inc., San Diego, CA). The first step in the workflow involved removing rRNA from the total RNA. Subsequently, the remaining mRNA was fragmented into small pieces using divalent cations at elevated temperatures. The cleaved RNA fragments were copied into first-strand cDNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and random primers. This was followed by synthesis of second-strand cDNA using DNA Polymerase I, RNase H, and dUTP. Next, these cDNA fragments went through an end repair process, involving the addition of a single 'A' base and ligation of the adapters. The products were purified and enriched by PCR to create a final cDNA

library. The libraries were quantified using KAPA Library Quantification kits for Illumina Sequencing platforms according to the qPCR Quantification Protocol Guide (KAPA BIOSYSTEMS, Woburn, MA) and qualified using TapeStation D1000 ScreenTape (Agilent Technologies, Santa Clara, CA, USA). Indexed libraries were then submitted to Illumina NovaSeq (Illumina, Inc., San Diego, CA), for paired-end sequencing (2×150 bp). Metagenomic analysis of viral pathogen detection was described in Fig. 5. Quality checking and trimming of short reads using Trim Galore (v.0.6.1) (Martin, 2011) with a Q30 threshold, followed by extraction of viral reads from the dataset using Deconseq (v0.4.3) (Schmieder and Edward., 2011) with 70% query coverage and 90% identity. Subsequently, the viral reads were assembled using the SPAdes assembler (v.3.15.1) (Bankevich *et al*., 2012), and the assembled contigs were annotated using BLAST+ (v.2.10.1) (Camacho *et al*., 2009) against the NCBI viral database with a rank1 cutoff and an e-value threshold of 1 x e^{-10}. Next, we calculated the alignment coverage with the genome of each viral species and contigs covering 90% of the full sequences of the identified viruses were analyzed.

Phylogenetic analysis

The obtained sequences were applied to a Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) database. The sequences were aligned using Clustal X (Ver 2.0) and examined with a similarity matrix. Phylogenetic analysis was performed using the neighbor-joining

method based on nucleotide alignments. Bootstrap analysis was conducted with 1,000 replicates using the MEGA version X.

Statistical analysis

All statistical methods were performed by SPSS (Version 25.0; IBM Corp., Armonk, NY, USA). The PCR results for each pathogen were recorded as positive or negative and categorized based on fecal scores. The association between fecal scores and each pathogen were compared using the Pearson's $\chi 2$ and linear by linear association.

3.3. Results

Sample collection

To investigate the prevalence of pathogens related to calf diarrhea, 810 fecal samples were collected from KIC on 15 farms in 2022 (Fig. 6). The collected feces were classified into two groups: 526 normal feces (267 with a fecal score of 0 and 259 with a fecal score of 1) and 284 diarrheic feces (178 with a fecal score of 2 and 106 with a fecal score of 3) (Table 24).

The association between fecal scores and each pathogens by PCR detection

To identify the association between fecal scores and each pathogen in calves, the presence of each pathogen in the 810 fecal samples was examined and described according to fecal scores (Table 25). As a result, BRV (14.0%, 113/810) was the most prevalent pathogen, followed by *C. parvum* (9.8%, 79/810), BVDV2 (4.9%, 40/810), BCV (3.2%, 26/810), BVDV1 (2.1%, 17/810), *Eimeria* spp. (1.9%, 15/810), and *Giardia* spp. (0.9%, 7/810). Among the seven pathogens, the detection rates of BRV ($p < 0.01$), *C. parvum* ($p < 0.001$), and *Eimeria* spp. ($p < 0.05$) increased as the fecal scores increased (Table 25).

Detection of viruses by next generation sequencing

To identify potential viruses causing calf diarrhea, NGS was conducted on a subset of 21 fecal samples with a score of 3 and no detection of the seven

aforementioned pathogens. The 21 samples were sequenced, generating 5,801,008–82,117,476 reads per sample (mean: 36,672,189; median: 22,283,010). After quality trimming, all reads with a Q30 or higher were considered high-quality, with over 95% of the reads meeting this threshold. Assembly was performed using the rnaviral pipeline of SPAdes, yielding contig sequences ranging from 87 to 65,044 (mean, 8,438; median, 3,732) for each sample. Contig sequences underwent BLAST analysis, resulting in a total of 3,798 results from all samples. After applying a filter with target virus coverage >= 90%, a final count of 178 viral contig sequences was obtained. Among these viral contigs, those associated with cattle or diarrhea were further analyzed. In 18 out of the 21 feces, more than one virus was detected, and metagenomic analysis revealed nearly complete sequences of bovine astrovirus (BAstV), bovine enterovirus (BEV), bovine kobuvirus (BKoV), bovine nebovirus (BNeV), bovine norovirus (BNoV), bovine boosepivirus B (BooV), bovine parechovirus (BParV), bovine torovirus (BToV), *C. parvum* virus 1 (CSpV1), and hunnivirus (Table 26).

Genomic characterization and phylogenetic analysis of bovine astrovirus

The complete genome size of BAstV is typically reported to be within the range of 6.4 to 7.3 kb. The BAstV RNA sequences were identified in 7 out of the 21 feces (Table 27), with genomic sizes varying between 6,052 and 6,288 nucleotides. Phylogenetic tree analysis based on complete genomic sequences showed that 1 feces was included in astrovirus group 2, while 1 feces was included in group 4, and

5 feces were included in group 5 (Fig. 7). Homology analysis revealed that the 1 obtained sequence belonging to astrovirus group 2 had 73.8% nucleotide identity with astrovirus group 2 isolate (LC047800) (Table A3), the 1 obtained sequence belonging to astrovirus group 4 had 73.2% nucleotide identity with astrovirus group 4 isolate (NC_037655) (Table A4), whereas the 5 obtained sequence belonging to astrovirus group 5 had 77.6-82.9% nucleotide identity with astrovirus group 4 isolate (LC047788) (Table A5).

Genomic characterization and phylogenetic analysis of bovine enterovirus

The complete genome size of BEV is typically reported to be within the range of 7.3 to 7.5 kb. The BEV genomic RNA sequences were identified in 2 out of the 21 feces (Table 27), with genomic sizes varying between 7,365 and 7,398 nucleotides. Phylogenetic tree analysis based on complete genomic sequences showed that 2 feces were included in BEV-F strain (Fig. 8). Homology analysis showed that 2 obtained sequences have 76.4% and 79.8% nucleotide characteristics with NCBI reference sequence of BEV-F strain isolate (NC_021220) (Table A6).

Genomic characterization and phylogenetic analysis of bovine kobuvirus

The complete genome size of BKoV is typically reported to be within the range of 8.2 to 8.4 kb. BKoV genomic RNA sequences were identified in 7 out of the 21 feces (Table 27), with genomic sizes varying between 8,293 and 8,441 nucleotides. Phylogenetic tree analysis based on complete genomic sequences

demonstrated that 5 fecal samples belonged to *Aichivirus* B, while 2 fecal samples belonged to *Aichivirus* D (Fig. 9). Homology analysis revealed that the 5 obtained sequences belonging to *Aichivirus* B had 89.9-90.2% nucleotide identity with *Aichivirus* B isolate (KT003671) (Table A7), whereas the 2 obtained sequences belonging to *Aichivirus* D had 79.4 and 83.6% nucleotide identity with *Aichivirus* D isolate (LC055960) (Table A8).

Genomic characterization and phylogenetic analysis of bovine nebovirus

The complete genome size of BNeV is typically reported to be within the range of 7.4 to 7.5 kb. The BNeV genomic RNA sequences were identified in 1 out of the 21 feces (Table 27), with genomic sizes 7,399 nucleotides. Phylogenetic tree analysis showed that 1 feces was included in Newbury strain (Fig. 10). Homology analysis showed that 1 obtained sequence have 81.5% nucleotide identity with BNeV isolate (NC_007916) (Table A9).

Genomic characterization and phylogenetic analysis of bovine norovirus

The complete genome size of BNoV is typically reported to be within the range of 7.2 to 7.3 kb. The BNoV genomic RNA sequences were identified in 2 out of the 21 feces (Table 27), with genomic sizes varying between 7,273 and 7,307 nucleotides. Phylogenetic tree analysis showed that 2 feces were included in Norovirus GIII type (Fig. 11). Homology analysis showed that 2 obtained sequences have 85.8% nucleotide identity with *Norovirus* GIII type isolate (NC_029645)

(Table A10).

Genomic characterization and phylogenetic analysis of bovine boosepivirus B

The complete genome size of BooV is typically reported to be within the range of 7.4 to 7.7 kb. The BooV RNA sequences were identified in 10 out of the 21 feces (Table 27), with genomic sizes varying between 7,613 and 7,750 nucleotides. Phylogenetic tree analysis based on complete genomic sequences showed that 10 feces were included in BooV B (Fig. 12). Homology analysis revealed that the 10 obtained sequences had 83.0-87.3% nucleotide identity with BooV B isolate (LC036579) (Table A11).

Genomic characterization and phylogenetic analysis of bovine parechovirus

The complete genome size of BParV is typically reported to be within the range of 7.7 to 7.8 kb. The BParV RNA sequences were identified in 1 out of the 21 feces (Table 27), with genomic sizes 7809 nucleotides. Phylogenetic tree analysis based on complete genomic sequences showed that they were not included in *Parechovirus* A to F, but classified with the previously reported sequences of other BParV (Fig. 13). Homology analysis showed that 1 obtained sequence have 86.3 % nucleotide identity with BParV isolate (BR001751) (Table A12).

Genomic characterization and phylogenetic analysis of bovine torovirus

The complete genome size of BToV is typically reported to be within the

range of 27.9 to 28.5 kb. The BToV RNA sequences were identified in 2 out of the 21 feces (Table 27), with genomic sizes 27,657 and 28,423 nucleotides. Phylogenetic tree analysis based on complete genomic sequences showed that 2 feces were most closely related to BToV from China (MN073058), but not closely related to BToV from Canada (AY427798) (Fig. 14). Homology analysis showed that 2 obtained sequences have 80.3% and 82.1% nucleotide identity with BToV isolate (AY427798) (Table A13).

Genomic characterization and phylogenetic analysis of *Cryptosporidium parvum* virus 1

These viruses contain two unrelated, linear dsRNA segments, 1.7 kbp (dsRNA1) and 1.4 kbp (dsRNA2), which are separately encapsidated, with dsRNA1 encoding the RNA-dependent RNA polymerase (RdRp) and dsRNA2 encoding the capsid protein (Nibert et al., 2009). The CSpV1 genomic RNA sequences were identified in 6 out of the 21 feces (Table 27), with dsRNA1 genomic sizes varying between 1,721 and 1,853 nucleotides and dsRNA2 genomic sizes varying between 1,486 and 1,539. Phylogenetic tree analysis based on dsRNA1 and dsRNA2 showed that the sequences obtained from this study were found to cluster together and distinct from other countries (Fig. 15, 16). Homology analysis revealed that the 6 obtained sequences have a 95.6-96.1% nucleotide identity with the NCBI reference sequence for CSpV1 dsRNA1 (RdRp) (NC_038843) (Table A14) and a 97.8-98.1% nucleotide identity with the NCBI reference sequence for CSpV1 dsRNA2 (capsid

protein) (NC_038844) (Table A15).

Genomic characterization and phylogenetic analysis of hunnivirus

The complete genome size of hunnivirus is typically reported to be within the range of 7.2 to 9.1 kb. The hunnivirus genomic RNA sequences were identified in 3 out of the 21 feces (Table 27), with genomic sizes varying between 7,565 and 7,597 nucleotides. Phylogenetic tree analysis showed that 3 feces were included in *Hunnivirus* A1 strain (Fig. 17). Homology analysis showed that 3 obtained sequences have 81.2-83.9% nucleotide identity with *Hunnivirus* A1 isolate (NC_018668) (Table A16).

3.4. Discussion

Infectious pathogens are a very important cause of diarrhea in calves, and many different pathogens are known to contribute to calf diarrhea (Cho and Yoon, 2014). However, despite many efforts, there is a significant lack of research on the pathogens that cause calf diarrhea. The study of pathogens using NGS has been widely used as a means to overcome these limitations and has also advanced the detection and analysis of pathogens. In this study, we aimed to determine the diagnostic capabilities of NGS by investigating a diverse range of viruses responsible for calf diarrhea, and to conduct epidemiological investigations of pathogens known to be the primary causes of calf diarrhea.

Compared with numerous studies on the prevalence of various pathogens associated with calf diarrhea worldwide (Nagai *et al*., 2015b; Kim *et al*., 2018; Lee *et al*., 2018; Isidan *et al*., 2019; Shi *et al*., 2019;), the previous research in the ROK has predominantly focused on major pathogens, including BRV, BCV, BVDV, *Cryptosporidium parvum*, *Giardia* spp., and *Eimeria* species (Kim *et al*., 2018; Lee *et al*., 2018; Chae *et al*., 2021; Kim *et al*., 2022). In this research, BRV was most detected in calf feces, followed by *C. parvum*, BVDV2, BCV, BVDV1, *Eimeria* spp., *Giardia* species. The prevalence of these pathogens exhibits considerable variation, which can be attributed to geographical differences, experimental methodologies, and timeframes, however, the findings of this study was consistent with those of

previous investigations, demonstrating no significant discrepancies. Bovine rotavirus, *C. parvum*, and *Eimeria* spp. demonstrated a statistically significant association with fecal scores. Both BRV and *C. parvum* have been identified as major causes of calf diarrhea not only in the ROK but also in other countries (Chae *et al*., 2021; Conrady *et al* 2021). Although commercial vaccines for BRV are available, controlling the disease remained challenging, and for *C. parvum*, no effective treatment is currently known worldwide. These pathogens persisted as ongoing issues, highlighting the need for more comprehensive and targeted research on effective preventive measures. In this study, *Eimeria* spp. showed a significant association with fecal scores; however, the low detection rate undermined the reliability of this finding. Future studies should be needed to identify the relationship between *Eimeria* spp. and calf diarrhea in greater detail.

In this study, 10 different viruses (BAstV, BEV, BKoV, BNeV, BNoV, BooV, BParV, BToV, CSpV1, and hunnivirus) were identified in calves with diarrhea using high-throughput sequencing. Bovine astrovirus (genus *Mamastrovirus*, family *Picornaviridae*) was known to occur several clinical signs in cattle including diarrhea and encephalitis (Woode *et al*., 1984; Janowski *et al*., 2021; Zhu *et al*., 2022). In this study, the sequences of BAstV were included in group 2, 4 and 5. Furthermore, group 1 BAstV was discovered from Korean indigenous cattle with non-suppurative meningoencephalitis in previous report in the ROK (Lee *et al*., 2021), suggesting that at least four distinct groups of BAstV have been identified in the ROK. To my best knowledge, this study is the first study to describe nearly complete genome of

group 2, 4, and 5 BAstV in calf diarrhea in the ROK.

Bovine enterovirus (genus *Enterovirus*, family *Picornaviridae*) has been reported the association with diarrhea (Li *et al*., 2012). The genus *Enterovirus* is classified into a total of 12 species and BEV is known to belong to species *Enterovirus* E and F (King *et al*., 2011). Further studies are needed to determine the clinical relevance of these findings, as there have been reports of bovine enterovirus (BEV) causing diarrhea and respiratory symptoms in cattle (Ley et al., 2002; Blas-Machado et al., 2011; Zhu et al., 2014). To my best knowledge, this study is the first study to describe nearly complete genome of BEV in calf diarrhea in the ROK.

Bovine kobuvirus (species *Aichivirus B*, genus *Kobuvirus*, family *Picornaviridae*), is known to cause calf diarrhea (Yamashita *et al*., 2003; Wang *et al*., 2020). *Aichivirus* strains was classified into six species (*Aichivirus* A to F) and obtained BKoV sequences in this study were included into *Aichivirus* B and D. In the previous reports, *Aichivirus* B and D was detected in various animals associated with diarrhea (Yamashita *et al*., 2003; Reuter *et al*., 2010; Smits *et al*., 2013; Otomaru *et al*., 2016). Further study should be performed to identify epidemiology, ecology, and roles of BKoV in calf diarrhea in the ROK, however, to my best knowledge, this study is the first study to describe nearly complete genome of BKoV in calf diarrhea in the ROK.

Bovine nebovirus (genus *Nebovirus*, family *Caliciviridae*), officially classified as a new genus in 2010, is also known to cause calf diarrhea (Cartens *et al*., 2009; Smiley *et al*., 2002). To date, three BNeV strains (Newbury-1, Dijon, and

Kirklareli strain) have been identified (Alkan *et al*., 2015; Kaplon *et al*., 2011; Oliver *et al*., 2006). The obtained BNeV sequence in this study was classified within the Newbury-1 strain, which was already previously reported in Korea (Park *et al*., 2008). Since BNeV has not been extensively investigated worldwide and underestimated their roles in calf diarrhea, ongoing research efforts are essential to enhance our understanding of this virus. To my best knowledge, this study is the first study to describe nearly complete genome of BNeV in calf diarrhea in the ROK.

Bovine norovirus (genus *Norovirus*, family *Caliciviridae*) is one of the major pathogens in calf diarrhea and viral infections in gnotobiotic calves showed diarrhea (Hall *et al*., 1984; Otto *et al*., 2011). Noroviruses are classified into seven genogroup (GI to GVII) and BNoV belongs to GIII (Di Felice *et al*., 2016), which was consistent with our findings. To my best knowledge, this study is the first study to describe nearly complete genome of BNoV GIII type in calf diarrhea in the ROK.

Boosepivirus (genus *Boosepivirus*, family *Picornaviridae*) was first identified in diarrhea from cattle in 2009 in Japan (Nagai *et al*., 2015a) and are currently reported in three groups: BooV A, B and C and only BooV B was detected in this study. Boosepivirus is a relatively recently discovered virus, with limited research conducted on its prevalence and impact (Nagai *et al*., 2015a; Wang *et al*., 2022), so further investigation should be needed to better understand the implications of BooV in calf diarrhea. To my best knowledge, this study is the first study to describe the existence and nearly complete genome of BooV B in calf diarrhea in the ROK.

Bovine parechovirus (genus *Parechovirus*, family *Picornaviridae*) was first identified in 2021 in the NCBI Sequence Read Archive (Kawasaki *et al*., 2021). The role of BParV in causing calf diarrhea remains uncertain; however, it is hypothesized to be a contributing factor based on previous findings that its initial detection in the digestive tracts of cattle and isolation from calf diarrhea (Kawasaki *et al*., 2021; Oba *et al*., 2023). The genus *Parechovirus* comprises six species, *Parechovirus* A-F, but BParV was not included in these species (Oba *et al*., 2023). Considering that BParV was initially identified in the ROK, it is essential to conduct further epidemiological investigations of the pathogen in other countries and examine its association with clinical manifestations.

Bovine torovirus (genus *Torovirus*, family *Tobaniviridae*) known to cause enteric and respiratory diseases in cattle, particularly affects young calves and reported worldwide (Hoet and Saif, 2004; Kirisawa *et al*., 2007). Based on phylogenetic analysis, the sequences of BToV obtained in this study were clustered distinct with torovirus from other animals, but further study should be performed since there has been reported the possibility of interspecies transmission of torovirus (Hoet, 2008; Ito *et al*., 2016). To my best knowledge, this study is the first study to describe nearly complete genome of BToV in calf diarrhea in the ROK.

Cryptosporidium parvum virus 1 (genus *Cryspovirus*, family *Partitiviridae*) was first identified from the cytoplasm of sporulated oocysts in North American *C. parvum* isolates (Khramtsov *et al*., 1997). Some impacts of CSpV1 on the pathogenicity of *C. parvum* were reported (Vainio *et al*., 2018; Deng *et al*., 2023).

The phylogenetic analysis conducted on CSpV dsRNA1 and dsRNA2 in this study indicated a tendency for these sequences to cluster according to their geographical origin, which was consistent with a previous study conducted in Japan that they revealed regional genetic variations (Murakoshi *et al*., 2016). Further investigation is required to characterize these genetic divergences and assess potential disparities in pathogenicity. To my best knowledge, this study is the first study to describe the existence and nearly complete genome of CSpV1 in calf diarrhea in the ROK.

Hunnivirus (genus *Hunnivirus* family *Picornaviridae*) have been reported in various animal species with including cattle, rats, and cats (Reuter *et al*., 2012; Firth *et al*., 2014; Lu *et al*., 2019) and was suspected to be associated with calf diarrhea (Işidan *et al*., 2019; Lu *et al*., 2019; Liu *et al*., 2022). Further research is necessary to elucidate the role of hunnivirus in these clinical manifestations. Based on phylogenetic analysis, hunniviruses identified in this study were classified as *Hunnivirus* A1, which included in bovine hunnivirus reported from Hungary. Due to the lack of existing research, the epidemiology, host range, and pathogenicity of hunnivirus remain undetermined. To my best knowledge, this study is the first study to describe the existence and nearly complete genome of hunnivirus in calf diarrhea in the ROK.

The traditional diagnostic methods for detecting pathogens in clinical samples have been constrained by the necessity of prior knowledge about potential infectious agents; without it, diagnosis is not feasible, however, NGS has emerged as a pivotal tool in surmounting this limitation. Despite various challenges, such as

the high cost of NGS and potential errors during experiments and analyses, a multitude of studies have substantiated the value of NGS as an innovative method for identifying previously unknown pathogens (Chiu *et al*., 2013). While downstream investigations of the viruses discovered in this study have not been performed yet, we identified 10 novel causative viral agents of calf diarrhea using the cutting-edge NGS technology, which might pose a previously unrecognized threat in the ROK. The finding of this study could potentially have substantial implications for the development of effective preventative measures and strategies to combat calf diarrhea, as well as contribute to the advancement and expansion of in-depth research in this field, ultimately enhancing our understanding of the etiology and epidemiology of this important veterinary health concern.

3.5. Conclusions

In this study, 7 pathogens associated with calf diarrhea were detected by PCR and the detection rates of BRV, *C. parvum*, and *Eimeria* spp. were significantly increased as fecal scores increased. Also, using high-throughput sequencing, the nearly complete genomic sequences of BAstV, BEV, BKoV, BNeV, BNoV, BooV, BParV, BToV, CSpV1, and hunnivirus were identified. This study represents the first investigation to identify the presence of BooV, CSpV1, and hunnivirus and it provides a comprehensive description of the nearly complete genomes of ten novel viruses associated with calf diarrhea in the ROK. The findings of this study would contribute to a better understanding of the epidemiology and molecular characteristics of calf diarrhea-associated pathogens in the ROK.

Table 23. Nucleotide sequences of polymerase chain reaction (PCR) primers/probes for pathogens causing calf diarrhea

Classifications	Microbial agents	PCR primers, probes	5'- nucleotide sequences - 3'	References
Viruses (conventional PCR)	bovine coronavirus	Forward	CTA GTA ACC AGG CTG ATG TCA ATA CC	Kim *et al.*, 2022
		Reverse	GGC GGA AAC CTA GTC GGA ATA	
	bovine rotavirus	Forward	TCA ACA TGG ATG TCC TGT ATT CCT	Solberg *et al.*, 2008
		Reverse	TCC CCC AGT TTG GAA TTC ATT	
Viruses (real-time PCR)	bovine viral diarrhea virus	Forward	CTC GAG ATG CCA TGT GGA C	Letellier *et al.*, 2003
		Reverse	CTC CAT GTG CCA TGT ACA GCA	
		BVD type 1 - Probe (FAM/BHQ1)	CAG CCT GAT AGG GTG CTG CAG AGG C	
		BVD type 2 - Probe (HEX/BHQ1)	CAC AGC CTG ATA GGG TGT AGC AGA GAC CTG	
Protozoa (real-time PCR)	*Cryptosporidium parvum*	Forward	CAA ATT GAT ACC GTT TGT CCT TCT GT	Guy *et al.*, 2003
		Reverse	GGC ATG TCG ATT CTA ATT CAG CT	
		Probe (HEX/BHQ1)	TGC CAT ACA TTG TTG TCC TGA CAA ATT GAA	
	Giardia spp.	Forward	CAT CCG CGA GGA GGT CAA	Guy *et al.*, 2003
		Reverse	GCA GCC ATG GTG TCG ATC T	
		Probe (FAM/BHQ1)	AAG TCC GCC GAC AAC ATG TAC CTA ACG A	
	Eimeria spp.	Forward	AAA GGA TGC AAA AGT CGT AAC AC	Thanthrige *et al.*, 2018
		Reverse	TGC AAT TCA CAA TGC GTA TCG	
		Probe (FAM/BHQ2)	TGT TTC TAC CCA CTA CAT CCA AC	

Table 24. The description of collected feces from 810 Korean indigenous calves (KIC) in 15 farms according to fecal scores

Farm IDs	Locations	No. of KIC				
		Fecal scores				
		0	1	2	3	Total
1	Anseong	13	16	7	1	37
2	Anseong	23	13	5	1	42
3	Anseong	18	26	28	26	98
4	Anseong	63	38	20	13	134
5	Yesan	5	13	10	5	33
6	Dangjin	39	44	29	8	120
7	Gongju	23	23	9	6	61
8	Cheongyang	12	13	6	3	34
9	Cheongyang	0	6	8	4	18
10	Nonsan	19	9	9	6	43
11	Buan	22	17	23	19	81
12	Namwon	14	19	12	5	50
13	Bongwha	6	4	1	1	12
14	Changnyeong	2	7	1	1	11
15	Sancheong	8	11	10	7	36
Total		267	259	178	106	810

Table 25. The detection rates of 7 pathogens in feces from 810 Korean indigenous calves (KIC) according to fecal scores

Pathogens	Fecal scores (No. of KIC, %)					P values	
	0 (n= 267)	1 (n=259)	2 (n=178)	3 (n=106)	Total (n=810)	Pe	Li
BRV	33 (12.4%)	30 (11.6%)	21 (11.8%)	29 (27.4%)	113 (14.0%)	<0.001	0.004
BCV	7 (2.6%)	8 (3.1%)	4 (2.2%)	7 (6.6%)	26 (3.2%)	0.189	0.170
BVDV1	3 (1.1%)	8 (3.1%)	3 (1.7%)	3 (2.8%)	17 (2.1%)	0.408	0.414
BVDV2	12 (4.5%)	12 (4.6%)	7 (3.9%)	9 (8.5%)	40 (4.9%)	0.336	0.277
C. parvum	12 (4.5%)	21 (8.1%)	28 (15.7%)	18 (17.0%)	79 (9.8%)	<0.001	<0.001
Eimeria spp.	2 (0.7%)	4 (1.5%)	5 (2.8%)	4 (3.8%)	15 (1.9%)	0.174	0.027
Giardia spp.	3 (1.1%)	3 (1.2%)	1 (0.6%)	0 (0.0%)	7 (0.9%)	0.662	0.257

Pe, Pearson's chi-square test; Li, linear by linear association; BRV, bovine rotavirus; BCV, bovine coronavirus; BVDV1, bovine viral diarrhea virus type 1; BVDV2, bovine viral diarrhea virus type 2; *C. parvum, Cryptosporidium parvum*.

Table 26. The information of detected viruses by next generation sequencing

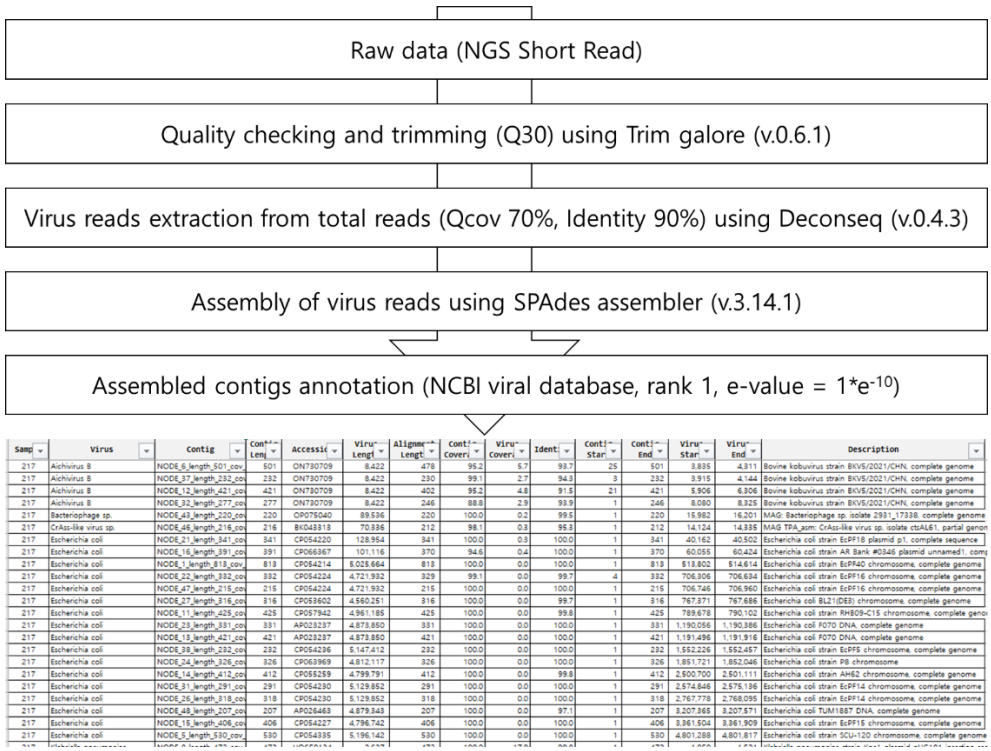
Sample IDs	Farm IDs	Locations	The list of detected virus by NGS
217	1	Anseong	BKoV, BooV
245	11	Buan	CSpV1
276	1	Anseong	BKoV, BooV
557	10	Nonsan	-
562	10	Nonsan	CSpV1
566	10	Nonsan	-
12151	10	Nonsan	BAstV, BToV
18707	6	Dangjin	BooV, BAstV, CSpV1
18897	4	Anseong	BKoV, BooV, BParV, BEV, hunnivirus
23358	5	Yesan	BKoV, BooV, hunnivirus
37284	10	Nonsan	BToV
48049	11	Buan	-
53954	2	Anseong	BKoV, BooV, BAstV, BEV
71346	11	Buan	BKoV, BooV, BAstV, CSpV1
73961	4	Anseong	BNoV
83561	15	Sancheong	BooV, BNeV, BNoV
85282	4	Anseong	BKoV, CSpV1, hunnivirus
86599	13	Bongwha	BAstV
88359	13	Bongwha	BooV
NA_4_475	6	Dangjin	BKoV, BooV, BAstV
NA_4_516	4	Anseong	CSpV1

BAstV, bovine astrovirus; BEV, bovine enterovirus; BKoV, bovine kobuvirus; BNeV, bovine nebovirus; BNoV, bovine norovirus; BooV, bovine boosepivirus; BParV, bovine parechovirus; BToV, bovine torovirus; CSpV1, *Cryptosporidium parvum* virus 1; -, not detected.

Table 27. The detection rates of viruses related with diarrhea in 21 Korean indigenous calves (KIC) by next generation sequencing

Virus	Description of detected viruses	No. of KIC (%)
BAstV	BAstV group 2	1 (4.8)
	BAstV group 4	1 (4.8)
	BAstV group 5	5 (24.8)
BEV	*Enterovirus* F	2 (9.5)
BKoV	*Aichivirus* B	5 (24.8)
	Aichivirus D	2 (9.5)
BNeV	BNeV Newbury strain	1 (4.8)
BNoV	*Norovirus* GIII	2 (9.5)
BooV	BooV B	10 (47.6)
BParV	-	1 (4.8)
BToV	-	2 (9.5)
CSpV1	-	6 (28.6)
Hunnivirus	*Hunnivirus* A1	3 (14.3)

BAstV, bovine astrovirus; BEV, bovine enterovirus; BKoV, bovine kobuvirus; BNeV, bovine nebovirus; BNoV, bovine norovirus; BooV, bovine boosepivirus; BParV, bovine parechovirus; BToV, bovine torovirus; CSpV1, *Cryptosporidium parvum* virus 1; -, No classification.



Samp	Virus	Contig	Contig Leng	Accessio	Virus Lengt	Alignme Lengt	Contig Coveri	Virus Coveri	Ident	Contig Star	Contig End	Virus Star	Virus End	Description
217	Aichivirus B	NODE_6_length_501_cov_	501	ON730709	8,422	478	95.2	5.7	93.7	25	501	3,835	4,311	Bovine kobuvirus strain BKV5/2021/CHN, complete genome
217	Aichivirus B	NODE_37_length_232_cov	232	ON730709	8,422	230	99.1	2.7	94.3	3	232	3,915	4,144	Bovine kobuvirus strain BKV5/2021/CHN, complete genome
217	Aichivirus B	NODE_12_length_421_cov	421	ON730709	8,422	402	95.2	4.8	91.5	21	421	5,906	6,306	Bovine kobuvirus strain BKV5/2021/CHN, complete genome
217	Aichivirus B	NODE_32_length_277_cov	277	ON730709	8,422	246	88.8	2.9	93.9	1	246	8,080	8,325	Bovine kobuvirus strain BKV5/2021/CHN, complete genome
217	Bacteriophage sp.	NODE_43_length_220_cov	220	OP075040	89,536	220	100.0	0.2	99.5	1	220	15,982	16,201	MAG: Bacteriophage sp. isolate 2931_17338, complete genome
217	CrAss-like virus sp.	NODE_46_length_216_cov	216	BK043313	70,336	212	98.1	0.3	95.3	1	212	14,124	14,335	MAG TPA_asm: CrAss-like virus sp. isolate ctsAL61, partial genon
217	Escherichia coli	NODE_21_length_341_cov	341	CP054220	128,954	341	100.0	0.3	100.0	1	341	40,162	40,502	Escherichia coli strain EcPF18 plasmid p1, complete sequence
217	Escherichia coli	NODE_16_length_391_cov	391	CP066367	101,116	370	94.6	0.4	100.0	1	370	60,055	60,424	Escherichia coli strain AR Bank #0346 plasmid unnamed1, comp
217	Escherichia coli	NODE_1_length_813_cov_	813	CP054214	5,025,664	813	100.0	0.0	100.0	1	813	513,802	514,614	Escherichia coli strain EcPF40 chromosome, complete genome
217	Escherichia coli	NODE_22_length_332_cov	332	CP054224	4,721,932	329	99.1	0.0	99.7	4	332	706,306	706,634	Escherichia coli strain EcPF16 chromosome, complete genome
217	Escherichia coli	NODE_47_length_215_cov	215	CP054224	4,721,932	215	100.0	0.0	100.0	1	215	706,746	706,960	Escherichia coli strain EcPF16 chromosome, complete genome
217	Escherichia coli	NODE_27_length_316_cov	316	CP053602	4,560,251	316	100.0	0.0	99.7	1	316	767,371	767,686	Escherichia coli BL21(DE3), chromosome, complete genome
217	Escherichia coli	NODE_11_length_425_cov	425	CP057942	4,961,185	425	100.0	0.0	99.8	1	425	789,678	790,102	Escherichia coli strain RHB09-C15 chromosome, complete genor
217	Escherichia coli	NODE_23_length_331_cov	331	AP023237	4,873,850	331	100.0	0.0	100.0	1	331	1,190,056	1,190,386	Escherichia coli F070 DNA, complete genome
217	Escherichia coli	NODE_13_length_421_cov	421	AP023237	4,873,850	421	100.0	0.0	100.0	1	421	1,191,496	1,191,916	Escherichia coli F070 DNA, complete genome
217	Escherichia coli	NODE_38_length_232_cov	232	CP054236	5,147,412	232	100.0	0.0	100.0	1	232	1,552,226	1,552,457	Escherichia coli strain EcPF5 chromosome, complete genome
217	Escherichia coli	NODE_24_length_326_cov	326	CP063969	4,812,117	326	100.0	0.0	100.0	1	326	1,851,721	1,852,046	Escherichia coli strain PB chromosome
217	Escherichia coli	NODE_14_length_412_cov	412	CP055259	4,799,791	412	100.0	0.0	99.8	1	412	2,500,700	2,501,111	Escherichia coli strain AHE2 chromosome, complete genome
217	Escherichia coli	NODE_31_length_291_cov	291	CP054230	5,129,852	291	100.0	0.0	100.0	1	291	2,574,846	2,575,136	Escherichia coli strain EcPF14 chromosome, complete genome
217	Escherichia coli	NODE_26_length_318_cov	318	CP054230	5,129,852	318	100.0	0.0	100.0	1	318	2,767,778	2,768,095	Escherichia coli strain EcPF14 chromosome, complete genome
217	Escherichia coli	NODE_48_length_207_cov	207	AP026463	4,879,343	207	100.0	0.0	97.1	1	207	3,207,365	3,207,571	Escherichia coli TUM1887 DNA, complete genome
217	Escherichia coli	NODE_15_length_406_cov	406	CP054227	4,796,742	406	100.0	0.0	100.0	1	406	3,961,504	3,961,909	Escherichia coli strain EcPF15 chromosome, complete genome
217	Escherichia coli	NODE_5_length_530_cov_	530	CP054335	5,196,142	530	100.0	0.0	100.0	1	530	4,801,288	4,801,817	Escherichia coli strain SCU-120 chromosome, complete genome

Fig. 5. Schematic diagram of the procedure for detecting viral sequences in calf diarrhea. Quality checking and trimming of short reads using Trim Galore (v.0.6.1) with a Q30 threshold, followed by extraction of viral reads from the dataset using Deconseq (v0.4.3) with 70% query coverage and 90% identity. Subsequently, the viral reads were assembled using the SPAdes assembler (v.3.15.1), and the assembled contigs were annotated using BLAST+ (v.2.10.1) against the NCBI viral database with a rank1 cutoff and an e-value threshold of 1 x e^{-10}.

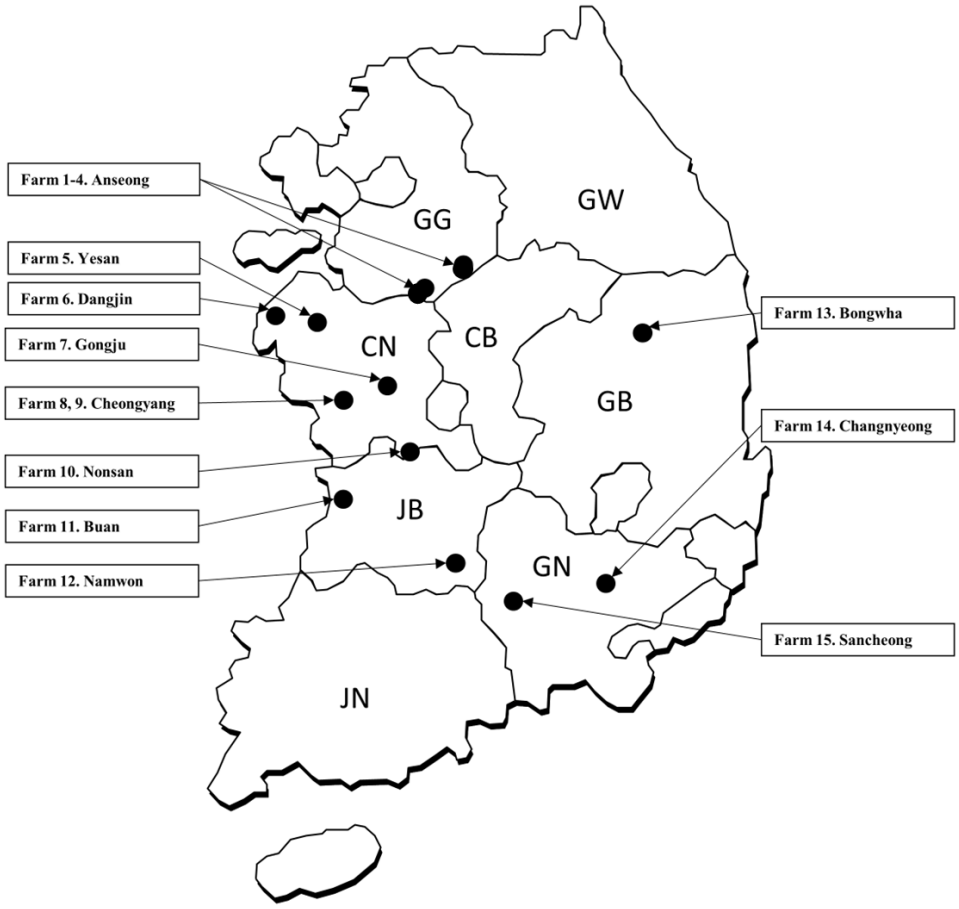


Fig. 6. The location of farms in this study. GG: Gyeonggi-do; GW: Gangwon-do; CB, Chungcheongbuk-do; CN, Chungcheongnam-do; GB, Gyeongsangbuk-do; GN, Gyeongsangnam-do; JB, Jeollabuk-do; JN, Jeollanam-do.

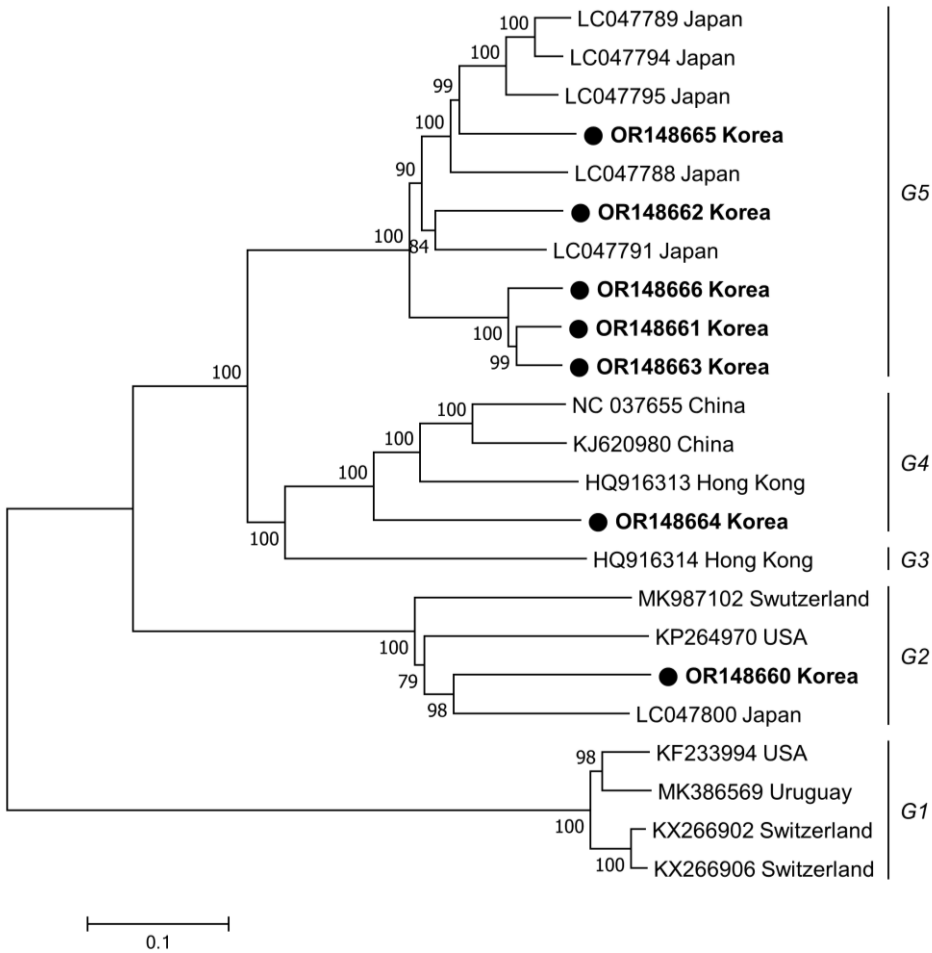


Fig. 7. Phylogenetic analysis of bovine astrovirus (BAstV). Neighbor-joining phylogenetic trees were based on complete genomic sequences of the obtained sequences in this study and other astroviruses. The bar represents a genetic distance. Numbers at nodes indicate bootstrap percentages obtained after 1000 bootstrap replicates. The obtained sequences in this study were marked with bold letter with a black circle (●).

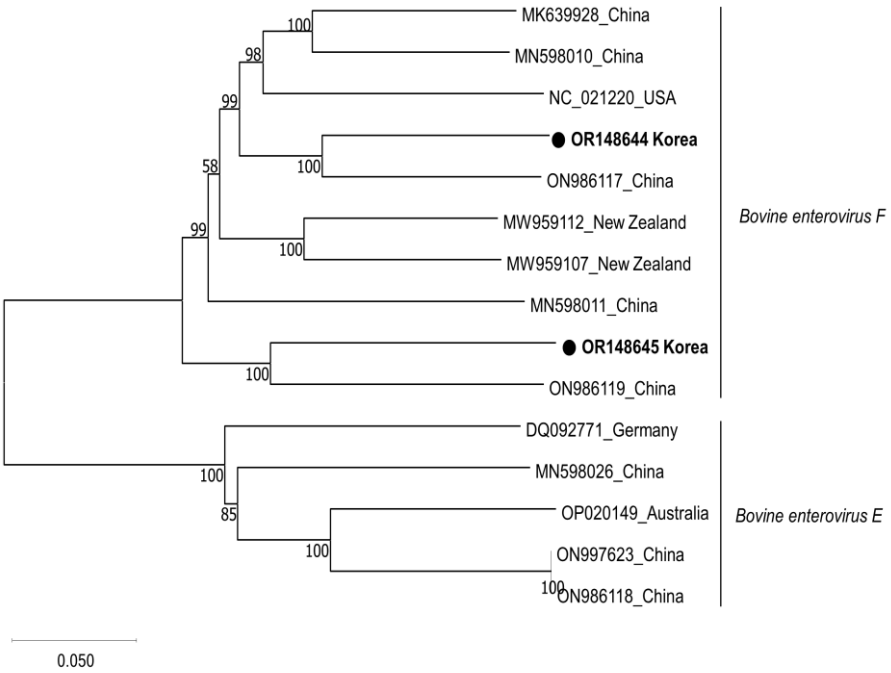


Fig. 8. Phylogenetic analysis of bovine enterovirus (BEV). Neighbor-joining phylogenetic trees were based on complete genomic sequences of the obtained sequences in this study and other BEVs. The bar represents a genetic distance. Numbers at nodes indicate bootstrap percentages obtained after 1000 bootstrap replicates. The obtained sequences in this study were marked with bold letter with a black circle (●).

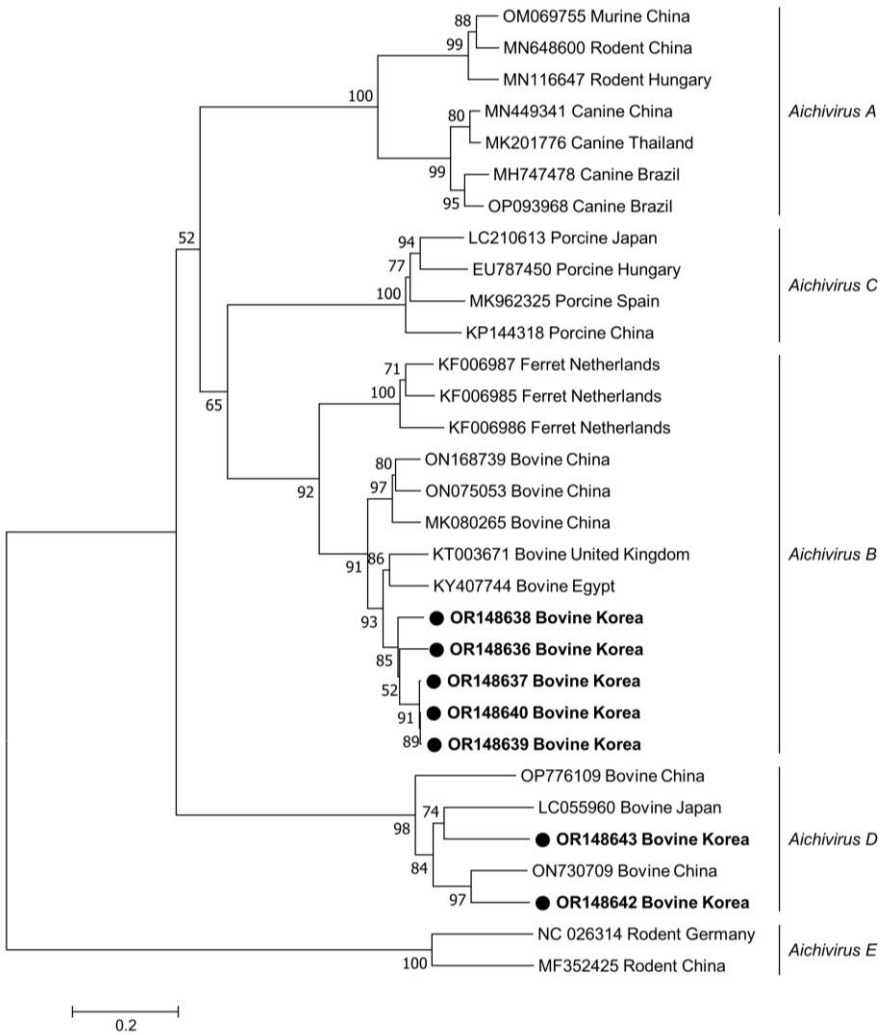


Fig. 9. Phylogenetic analysis of bovine kobuvirus (BKoV). Neighbor-joining phylogenetic trees were based on complete genomic sequences of the obtained sequences in this study and other aichiviruses. The bar represents a genetic distance. Numbers at nodes indicate bootstrap percentages obtained after 1000 bootstrap replicates. The obtained sequences in this study were marked with bold letter with a black circle (●).

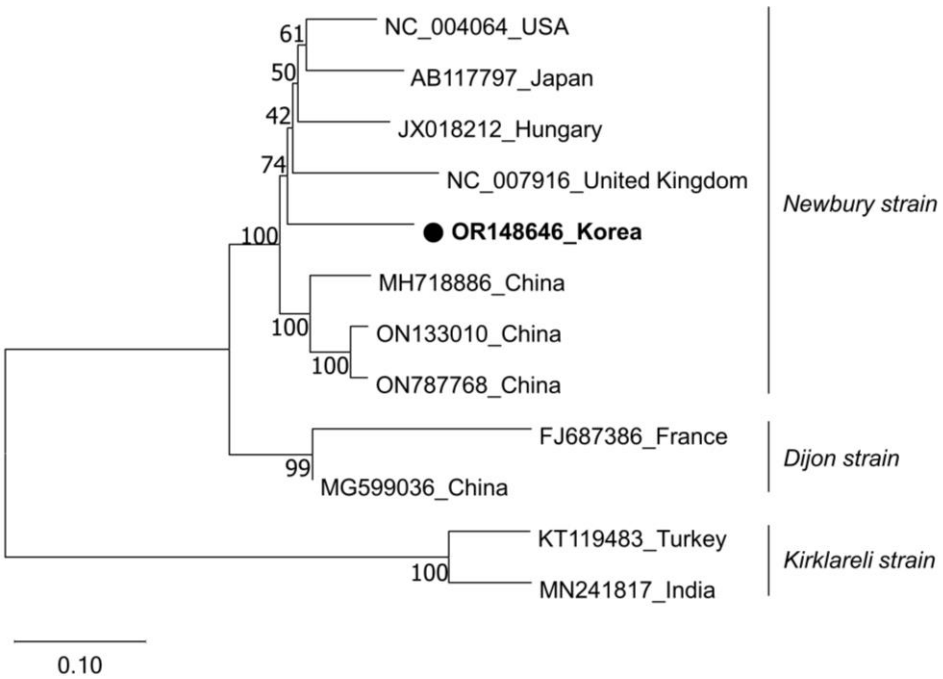


Fig. 10. Phylogenetic analysis of bovine nebovirus (BNeV). Neighbor-joining phylogenetic trees were based on complete genomic sequences of the obtained sequence in this study and other BNeVs. The bar represents a genetic distance. Numbers at nodes indicate bootstrap percentages obtained after 1000 bootstrap replicates. The obtained sequences in this study were marked with bold letter with a black circle (●).

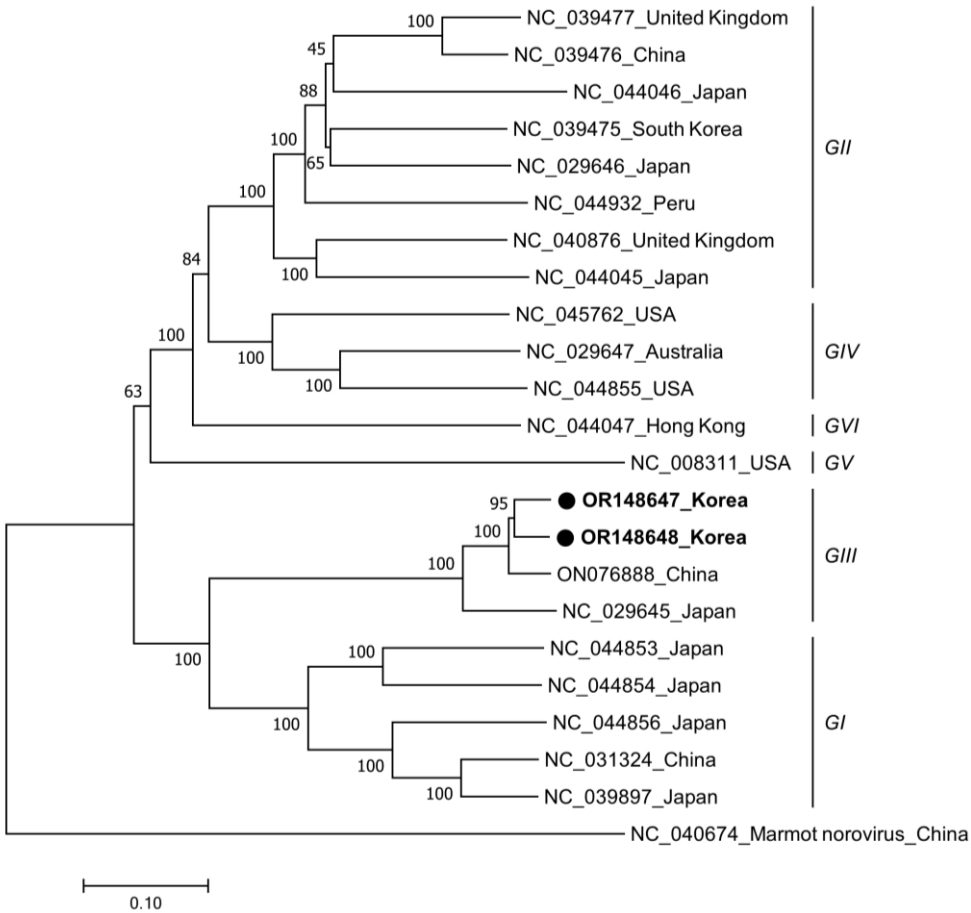


Fig. 11. Phylogenetic analysis of bovine norovirus (BNoV). Neighbor-joining phylogenetic trees were based on complete genomic sequences of the obtained sequence in this study and other noroviruses. The bar represents a genetic distance. Numbers at nodes indicate bootstrap percentages obtained after 1000 bootstrap replicates. The obtained sequences in this study were marked with bold letter with a black circle (●).

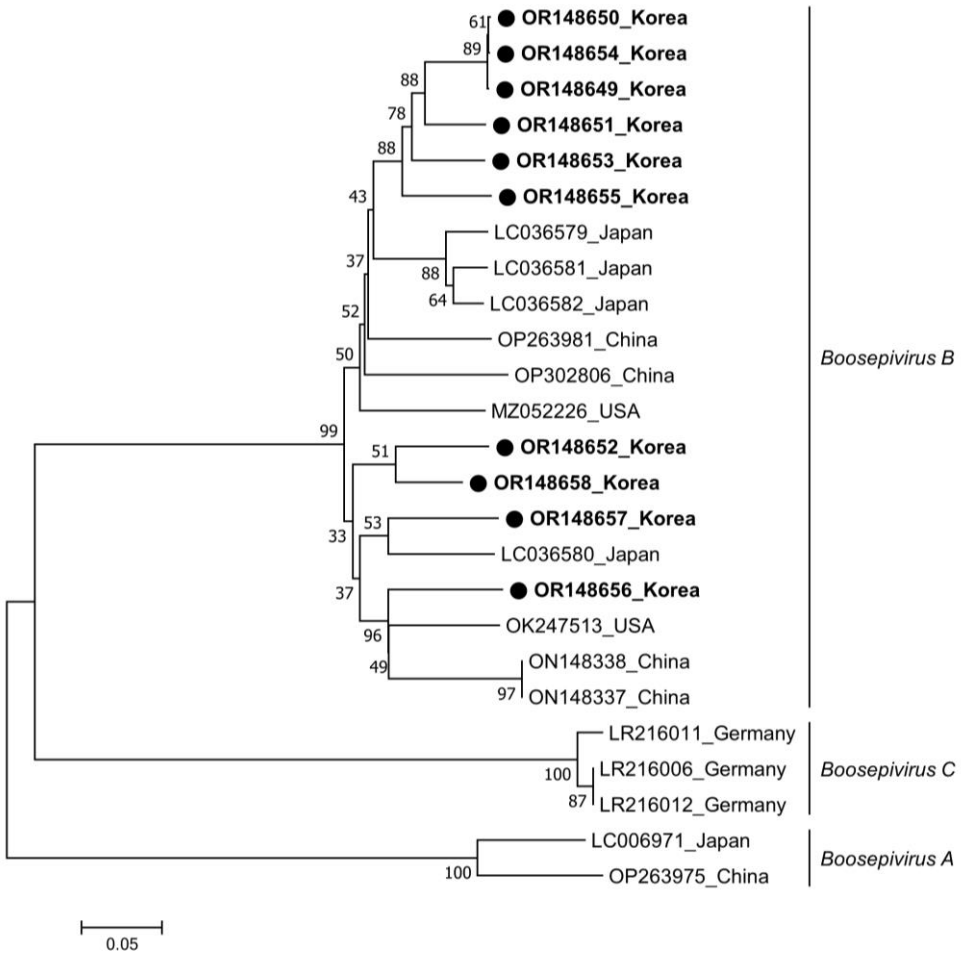


Fig. 12. Phylogenetic analysis of bovine boosepivirus (BooV). Neighbor-joining phylogenetic trees were based on complete genomic sequences of the obtained sequences in this study and other boosepiviruses. The bar represents a genetic distance. Numbers at nodes indicate bootstrap percentages obtained after 1000 bootstrap replicates. The obtained sequences in this study were marked with bold letter with a black circle (●).

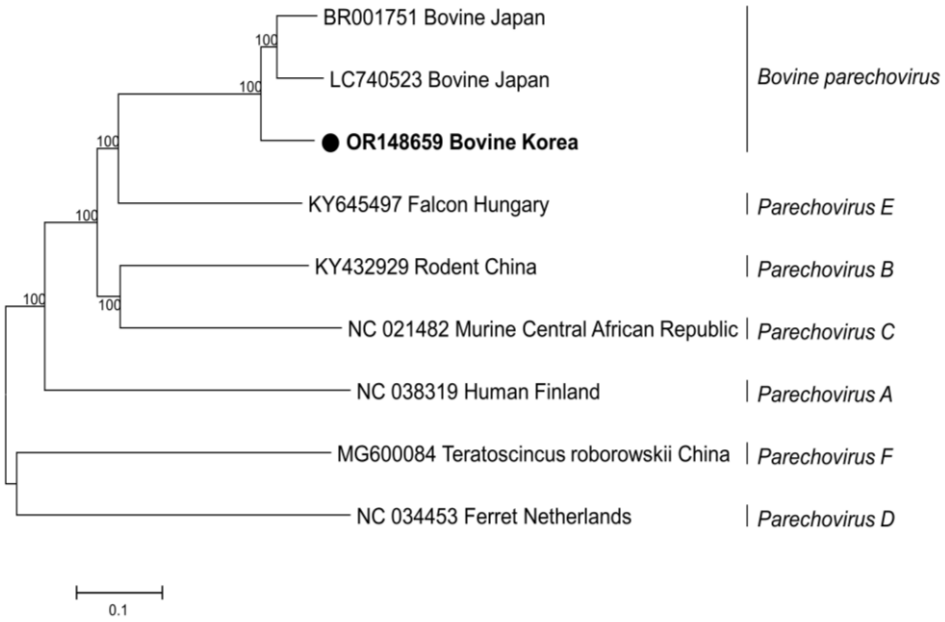


Fig. 13. Phylogenetic analysis of bovine parechovirus (BParV). Neighbor-joining phylogenetic trees were based on complete genomic sequences of the obtained sequence in this study and other parechoviruses. The bar represents a genetic distance. Numbers at nodes indicate bootstrap percentages obtained after 1000 bootstrap replicates. The obtained sequences in this study were marked with bold letter with a black circle (●).

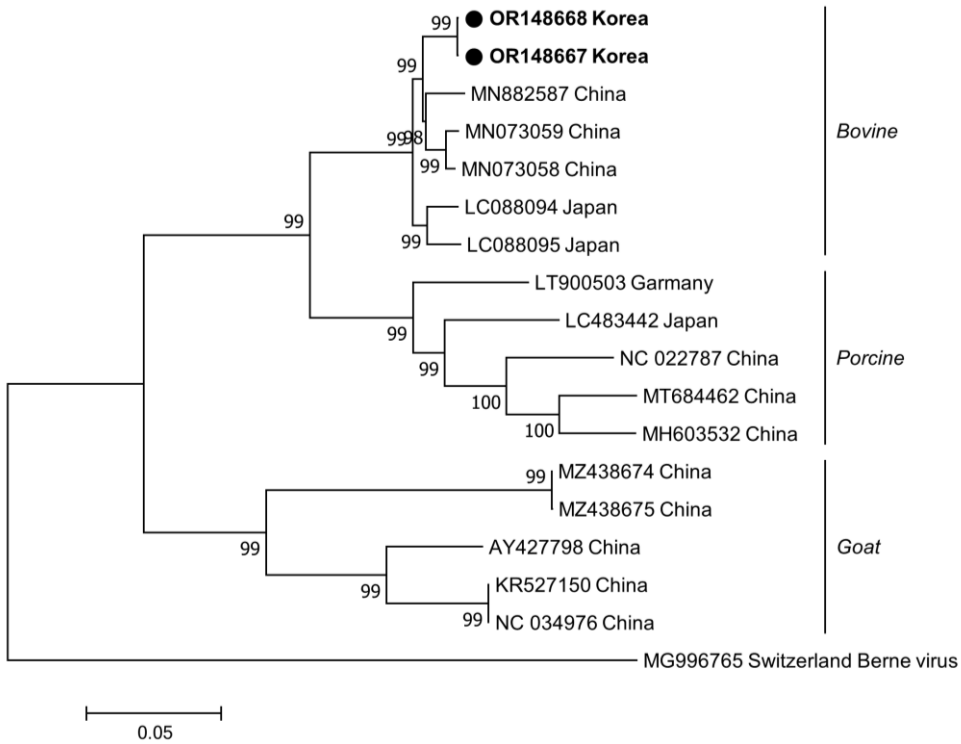


Fig. 14. Phylogenetic analysis of bovine torovirus (BToV). Neighbor-joining phylogenetic trees were based on complete genomic sequences of the obtained sequences in this study and other toroviruses. The bar represents a genetic distance. Numbers at nodes indicate bootstrap percentages obtained after 1000 bootstrap replicates. The obtained sequences in this study were marked with bold letter with a black circle (●).

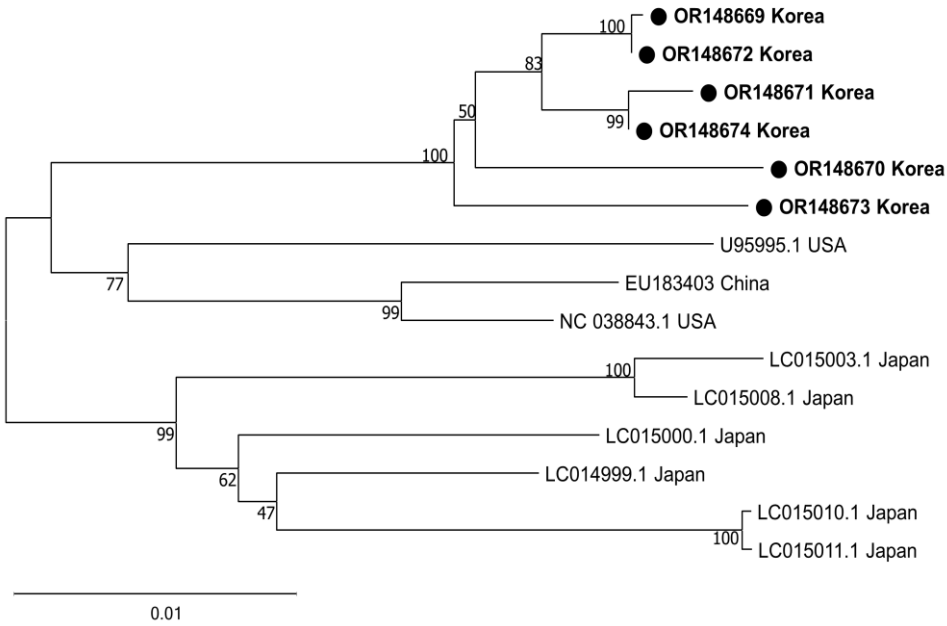


Fig. 15. Phylogenetic analysis of dsRNA1 of *Cryptosporidium parvum* virus 1 (CSpV1). Neighbor-joining phylogenetic trees were based on complete genomic sequences of the obtained sequences in this study and other CSpV1 dsRNA1. The bar represents a genetic distance. Numbers at nodes indicate bootstrap percentages obtained after 1000 bootstrap replicates. The obtained sequences in this study were marked with bold letter with a black circle (●).

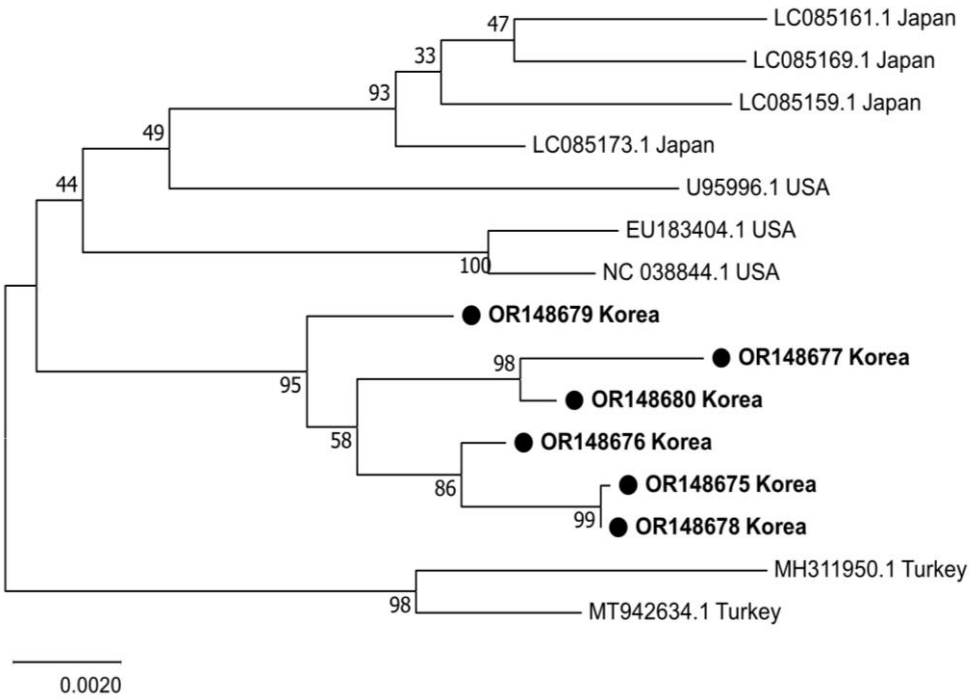


Fig. 16. Phylogenetic analysis of dsRNA2 of *Cryptosporidium parvum* virus 1 (CSpV1). Neighbor-joining phylogenetic trees were based on complete genomic sequences of the obtained sequences in this study and other CSpV1 dsRNA2**.** The bar represents a genetic distance. Numbers at nodes indicate bootstrap percentages obtained after 1000 bootstrap replicates. The obtained sequences in this study were marked with bold letter with a black circle (●).

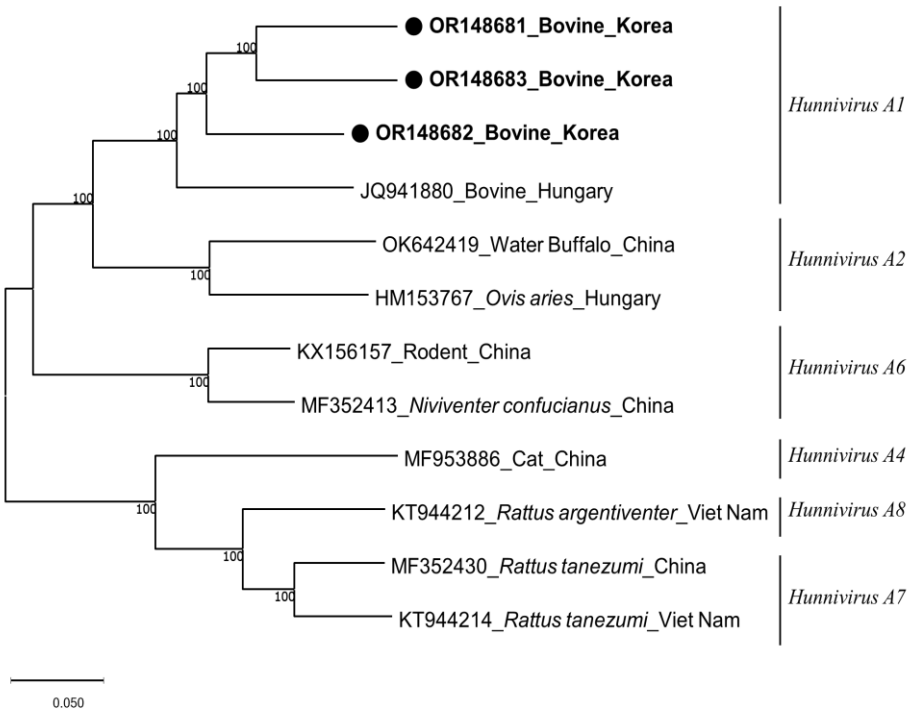


Fig. 17. Phylogenetic analysis of hunnivirus. Neighbor-joining phylogenetic trees were based on complete genomic sequences of the obtained sequence in this study and other hunniviruses. The bar represents a genetic distance. Numbers at nodes indicate bootstrap percentages obtained after 1000 bootstrap replicates. The obtained sequences in this study were marked with bold letter with a black circle (●).

General Conclusions

There have been studied about calf diarrhea by numerous researchers, however, calf diarrhea is a still major concern in cattle industries in the ROK and worldwide. As calf diarrhea is a multifactorial disease, it should be approached in the consideration of the host status, causes, and methods for prevention. In this study, we evaluated the host status in calf diarrhea with hematology, serum biochemistry, and acute phase proteins, identified the major causes of calf diarrhea, and novel viruses associated with calf diarrhea in the ROK.

1. The host status in calves having diarrhea was evaluated using hematology, serum biochemistry, and acute phase proteins. Levels of BUN, Glu, Na, K, Fib, and Hp showed significant difference and linear associations according to fecal scores. Furthermore, after ROC analysis, the optimal cut-off values were calculated for BUN, K, Fib, and Hp. They were as follows: BUN (9.50 mg/dL), K (5.75 mmol/dL), Fib (650.0 mg/dL), and Hp (12.5 mg/dL). This study was the first to calculate RI of acute phase proteins in KIC and application to calf diarrhea.

2. After evaluation of host status in calf diarrhea, seven major pathogens causing calf diarrhea (*Eimeria* spp., BRV, BCV, *C. parvum*, BVDV, *E. coli* K99, and *Salmonella* spp.) and the disease caused by them were investigated. Multiple pathogens were detected in association with calf diarrhea; *C. parvum* and BRV appeared to be the primary pathogens significantly influencing calf diarrhea in

KIC. BCV showed significant association with BRV and *C. parvum*. The findings of this study highlighted the importance of viral pathogens in calf diarrhea.

3. Furthermore, 7 pathogens associated with calf diarrhea were detected by PCR and the detection rates of BRV, *C. parvum*, and *Eimeria* spp. were significantly increased as fecal scores increased. Also, using high-throughput sequencing, the nearly complete genomic sequences of BAstV, BEV, BKoV, BNeV, BNoV, BooV, BParV, BToV, CSpV1, and hunnivirus were identified. This study represents the first investigation to identify the presence of BooV, CSpV1, and hunnivirus and provide the descriptions of nearly complete genomes of ten novel viruses associated with calf diarrhea in the ROK. The findings of this study would contribute to a better understanding of the epidemiology and molecular characteristics of calf diarrhea-associated pathogens in the ROK.

Bovine medicine in the ROK has emphasized on both clinical and preventive veterinary medicine compared to medicinal practices for other industrial animals. This study focused on both clinical and preventive veterinary medicine for calf diarrhea. The finding of this study could potentially have substantial implications for the development of effective preventative measures and strategies to combat calf diarrhea, as well as contribute to the advancement and expansion of in-depth research in this field, ultimately enhancing our understanding of the etiology and epidemiology of this important veterinary health concern.

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Appendices

Table A1. Fecal scores and seasons description of feces from 544 Korean indigenous calves (KIC) in each of 10 farms

Farms	Seasons	Fecal scores (No. of KIC, %)			
		0	1	2	3
1	Spring (n=11)	2 (18.2)	4 (36.4)	5 (45.5)	0 (0.0)
	Summer (n=4)	2 (50.0)	0 (0.0)	2 (50.0)	0 (0.0)
	Autumn (n=4)	2 (50.0)	0 (0.0)	1 (25.0)	1 (25.0)
	Subtotal (n=19)	**6 (31.6)**	**4 (21.1)**	**8 (42.1)**	**1 (5.3)**
2	Spring (n=58)	17 (29.3)	22 (37.9)	12 (20.7)	7 (12.1)
	Summer (n=33)	2 (6.1)	5 (15.2)	10 (30.3)	16 (48.5)
	Autumn (n=19)	7 (38.9)	4 (22.2)	5 (27.8)	2 (11.1)
	Subtotal (n=109)	**26 (23.9)**	**31 (28.4)**	**27 (24.8)**	**25 (22.9)**
3	Spring (n=95)	28 (29.5)	37 (38.9)	22 (23.2)	8 (8.4)
	Summer (n=39)	5 (12.8)	22 (56.4)	5 (12.8)	7 (17.9)
	Autumn (n=41)	15 (36.6)	17 (41.5)	5 (12.2)	4 (9.8)
	Subtotal (n=175)	**48 (27.4)**	**76 (43.4)**	**32 (18.3)**	**19 (10.9)**
4	Spring (n=12)	1 (8.3)	0 (0.0)	9 (75.0)	2 (16.7)
	Summer (n=4)	1 (25.0)	1 (25.0)	0 (0.0)	2 (50.0)
	Autumn (n=13)	1 (7.7)	1 (7.7)	5 (38.5)	6 (46.2)
	Subtotal (n=29)	**3 (10.3)**	**2 (6.9)**	**14 (48.3)**	**10 (34.5)**
5	Spring (n=4)	1 (25.0)	3 (75.0)	0 (0.0)	0 (0.0)
	Summer (n=5)	2 (40.0)	3 (60.0)	0 (0.0)	0 (0.0)
	Autumn (n=0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Subtotal (n=9)	**3 (33.3)**	**6 (66.7)**	**0 (0.0)**	**0 (0.0)**

Table A1. Fecal scores and seasons description of feces from 544 Korean indigenous calves (KIC) in each of 10 farms (Continued)

Farms	Seasons	Fecal scores (No. of KIC, %)			
		0	1	2	3
6	Spring (n=13)	4 (30.8)	7 (53.8)	0 (0.0)	2 (15.4)
	Summer (n=16)	5 (31.3)	2 (12.5)	4 (25.0)	5 (31.3)
	Autumn (n=7)	2 (28.6)	1 (14.3)	3 (42.9)	1 (14.3)
	Subtotal (n=36)	**11 (30.6)**	**10 (27.8)**	**7 (19.4)**	**8 (22.2)**
7	Spring (n=7)	3 (42.9%)	4 (57.1)	0 (0.0)	0 (0.0)
	Summer (n=1)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Autumn (n=0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Subtotal (n=8)	**4 (50.0)**	**4 (50.0)**	**0 (0.0)**	**0 (0.0)**
8	Spring (n=49)	20 (40.8)	18 (36.7)	6 (12.2)	5 (10.2)
	Summer (n=15)	2 (13.3)	5 (33.3)	3 (20.0)	5 (33.3)
	Autumn (n=21)	10 (47.6)	5 (23.8)	5 (23.8)	1 (4.8)
	Subtotal (n=85)	**32 (37.6)**	**28 (32.9)**	**14 (16.5)**	**11 (12.9)**
9	Spring (n=28)	7 (25.0)	10 (35.7)	5 (17.9)	6 (21.4)
	Summer (n=10)	3 (30.0)	2 (20.0)	3 (30.0)	2 (20.0)
	Autumn (n=23)	7 (30.4)	7 (30.4)	5 (21.7)	4 (17.4)
	Subtotal (n=61)	**17 (27.9)**	**19 (31.1)**	**13 (21.3)**	**12 (19.7)**
10	Spring (n=5)	0 (0.0)	5 (100.0)	0 (0.0)	0 (0.0)
	Summer (n=5)	2 (40.0)	1 (20.0)	1 (20.0)	1 (20.0)
	Autumn (n=3)	1 (33.3)	1 (33.3)	0 (0.0)	1 (33.3)
	Subtotal (n=13)	**3 (23.1)**	**7 (53.8)**	**1 (7.7)**	**2 (15.4)**

Table A2. Fecal scores and pathogens presence of feces from 544 Korean indigenous calves (KIC) in each of 10 farms

Farms	Pathogen presence	Fecal scores (No. of KIC, %)			
		0	1	2	3
1	Negative (n=12)	5 (41.7)	4 (33.3)	3 (25.0)	0 (0.0)
	Positive (n=7)	1 (14.3)	0 (0.0)	5 (71.4)	1 (14.3)
	Subtotal (n=19)	**6 (31.6)**	**4 (21.1)**	**8 (42.1)**	**1 (5.3)**
2	Negative (n=53)	17 (32.1)	18 (34.0)	10 (18.9)	8 (15.1)
	Positive (n=56)	9 (16.1)	13 (23.2)	17 (30.4)	17 (30.4)
	Subtotal (n=109)	**26 (23.9)**	**31 (28.4)**	**27 (24.8)**	**25 (22.9)**
3	Negative (n=101)	28 (27.7)	42 (41.6)	23 (22.8)	8 (7.9)
	Positive (n=74)	20 (27.0)	34 (45.9)	9 (12.2)	11 (14.9)
	Subtotal (n=175)	**48 (27.4)**	**76 (43.4)**	**32 (18.3)**	**19 (10.9)**
4	Negative (n=9)	3 (33.3)	2 (22.2)	4 (44.4)	0 (0.0)
	Positive (n=20)	0 (0.0)	0 (0.0)	10 (50.0)	10 (50.0)
	Subtotal (n=29)	**3 (10.3)**	**2 (6.9)**	**14 (48.3)**	**10 (34.5)**
5	Negative (n=8)	2 (25.0)	6 (75.0)	0 (0.0)	0 (0.0)
	Positive (n=1)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Subtotal (n=9)	**3 (33.3)**	**6 (66.7)**	**0 (0.0)**	**0 (0.0)**

Table A2. Fecal scores and pathogens presence of feces from 544 Korean indigenous calves (KIC) in each of 10 farms (Continued)

Farms	Pathogen presence	Fecal scores (No. of KIC calves, %)			
		0	1	2	3
6	Negative (n=29)	8 (27.6)	9 (31.0)	6 (20.7)	6 (20.7)
	Positive (n=7)	3 (42.9)	1 (14.3)	1 (14.3)	2 (28.6)
	Subtotal (n=36)	**11 (30.6)**	**10 (27.8)**	**7 (19.4)**	**8 (22.2)**
7	Negative (n=7)	4 (57.1)	3 (42.9)	0 (0.0)	0 (0.0)
	Positive (n=1)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)
	Subtotal (n=8)	**4 (50.0)**	**4 (50.0)**	**0 (0.0)**	**0 (0.0)**
8	Negative (n=55)	17 (30.9)	17 (30.9)	12 (21.8)	9 (16.4)
	Positive (n=30)	15 (50.0)	11 (36.7)	2 (6.7)	2 (6.7)
	Subtotal (n=85)	**32 (37.6)**	**28 (32.9)**	**14 (16.5)**	**11 (12.9)**
9	Negative (n=32)	9 (28.1)	13 (40.6)	4 (12.5)	6 (18.8)
	Positive (n=29)	8 (27.6)	6 (20.7)	9 (31.0)	6 (20.7)
	Subtotal (n=61)	**17 (27.9)**	**19 (31.1)**	**13 (21.3)**	**12 (19.7)**
10	Negative (n=8)	3 (37.5)	3 (37.5)	0 (0.0)	2 (25.0)
	Positive (n=5)	0 (0.0)	4 (80.0)	1 (20.0)	0 (0.0)
	Subtotal (n=13)	**3 (23.1)**	**7 (53.8)**	**1 (7.7)**	**2 (15.4)**

Table A3. The nucleotide identities of the obtained sequences of bovine astrovirus compared to bovine astrovirus group 2 isolate (LC047800)

Sample IDs	Nucleotide identities (%)		
	Complete genome	ORF1ab	ORF2
12151	73.8	87.34	51.45
18707	47.9	-	-
53954	-	-	-
71346	49.3	-	-
73961	-	-	-
86599	48.5	-	-
NA_4_475	49.2	-	-

-, Nucleotide similarity less than 25%.

Table A4. The nucleotide identities of the obtained sequences of bovine astrovirus compared to bovine astrovirus group 4 isolate (NC_037655)

Sample IDs	Nucleotide identities (%)		
	Complete genome	ORF1ab	ORF2
12151	47.4	-	-
18707	60.0	71.0	46.4
53954	60.2	71.0	46.9
71346	59.3	70.2	-
73961	73.2	88.8	49.2
86599	61.4	71.7	49.0
NA_4_475	59.6	70.6	-

-, Nucleotide similarity less than 25%.

Table A5. The nucleotide identities of the obtained sequences of bovine astrovirus compared to bovine astrovirus group 5 isolate (LC047788)

Sample IDs	Nucleotide identities (%)		
	Complete genome	ORF1ab	ORF2
12151	47.8	-	-
18707	78.7	84.8	68.9
53954	79.3	86.6	67.1
71346	78.1	85.1	66.6
73961	60.4	71.3	-
86599	82.9	90.9	70.5
NA_4_475	77.6	84.3	66.7

-, Nucleotide similarity less than 25%.

Table A6. The nucleotide identities of the obtained sequences of bovine enterovirus compared to bovine enterovirus isolate (NC_021220)

Sample IDs	Nucleotide identities (%)										
	Complete genome	**VP4**	**VP2**	**VP3**	**VP1**	**2A**	**2B**	**2C**	**3A**	**3C**	**RdRp**
18897	79.8	80.7	73.7	74.1	67.9	70.0	77.1	83.3	79.0	84.3	88.1
53954	76.4	75.4	75.0	75.0	57.7	74.0	79.1	81.0	79.0	85.4	86.5

Table A7. The nucleotide identities of the obtained sequences of bovine kobuvirus compared to Aichivirus B isolate (KT003671)

Sample IDs	Nucleotide identities (%)											
	Complete genome	L	VP0	VP3	VP1	2A	2B	2C	3A	3B	3C	3D
00276	90.0	86.6	90.0	87.0	87.4	91.3	88.3	91.8	90.8	96.7	90.3	93.3
18897	89.9	86.5	89.1	88.6	87.3	92.5	87.7	93.2	90.1	85.6	92.5	92.3
23358	-	-	55.2	63.0	50.4	53.9	-	58.7	-	-	-	64.9
71346	90.1	86.3	88.6	88.3	86.5	92.0	87.5	92.7	92.2	96.7	91.7	92.3
85282	90.2	85.9	89.1	88.6	87.6	82.0	87.7	93.2	90.1	95.6	52.5	92.1
NA_4_475	90.2	86.1	89.2	88.8	87.6	92.3	87.7	93.2	90.1	95.6	92.4	92.1
53954	-	-	57.5	59.5	54.4	55.8	-	59.5	-	-	-	62.0

-, Nucleotide similarity less than 25%.

Table A8. The nucleotide identities of the obtained sequences of bovine kobuvirus compared to Aichivirus D isolate (LC055960)

Sample IDs	Nucleotide identities (%)											
	Complete genome	L	VP0	VP3	VP1	2A	2B	2C	3A	3B	3C	3D
00276	-	-	57.5	60.7	54.1	56.9	-	57.0	-	-	55.2	65.4
18897	-	-	57.1	61.9	54.2	57.9	-	58.3	-	-	55.9	65.4
23358	79.4	82.7	64.0	70.3	62.1	94.3	81.7	81.2	93.0	85.4	85.5	91.3
71346	-	-	56.3	62.0	54.1	55.9	-	57.9	-	-	56.1	65.1
85282	-	-	57.3	61.9	54.2	57.9	-	58.3	-	-	55.9	65.4
NA_4_475	-	-	57.3	61.9	54.2	58.1	-	58.3	-	-	55.7	65.4
53954	83.6	85.8	82.0	74.6	75.3	92.1	79.1	81.9	91.2	83.3	84.2	90.8

-, Nucleotide similarity less than 25%.

Table A9. The nucleotide identities of the obtained sequences of bovine nebovirus compared to bovine nebovirus isolate (NC007916)

Sample IDs	Nucleotide identities (%)		
	Complete genome	polyprotein	ORF2
83561	81.5	81.6	87.5

Table A10. The nucleotide identities of the obtained sequences of bovine norovirus compared to norovirus GIII type isolate (NC_029645)

Sample IDs	Nucleotide identities (%)								
	Complete genome	p48	NTPase	p22	Vpg	Pro	RdRp	VP1	VP2
73961	85.8	84.1	85.3	85.3	87.4	87.3	88.1	89.9	87.0
83561	85.8	83.5	85.8	85.1	86.1	87.5	88	85.9	85.3

Table A11. The nucleotide identities of the obtained sequences of boosepivirus compared to boosepivirus B isolate (LC036579)

Sample IDs	Nucleotide identities (%)											
	Complete genome	L	VP4	VP2	VP3	VP1	2A	2B	2C	3A	3C	3D
00217	87.3	89.6	86.1	85.0	87.6	85.1	87.9	86.7	87.5	90.0	88.6	88.6
00276	87.1	89.1	86.1	85.1	87.6	85.4	87.3	86.7	87.4	90.0	88.4	88.6
18707	86.8	90.7	86.6	85.1	88.4	83.6	87.6	85.8	86.6	86.5	89.1	87.8
18897	83.0	88.0	75.1	77.6	80.5	72.7	85.2	83.6	83.7	86.8	88.6	87.7
23358	86.8	88.5	87.6	83.8	87.9	84.5	87.3	85.2	87.4	87.6	88.8	87.7
53954	87.2	89.6	86.1	85.0	87.3	85.6	87.6	86.7	87.4	90.0	88.6	88.6
71346	86.8	88.5	83.1	85.5	86.3	84.6	86.7	87.3	85.0	90.2	88.8	88.9
83561	83.9	86.9	77.1	77.2	78.3	74.1	86.5	84.6	85.7	90.2	89.9	88.3
88359	83.3	89.1	79.1	78.6	82.7	73.1	84.3	85.2	85.8	87.3	89.0	87.0
NA_4_475	86.0	89.6	84.6	84.1	86.7	84.8	86.5	83.6	83.8	86.2	88.6	87.8

Table A12. The nucleotide identities of the obtained sequences of bovine parechovirus compared to bovine parechovirus isolate (BR001751)

Sample IDs	Nucleotide identities (%)	
	Complete genome	Polyprotein
18897	86.3	88.4

Table A13. The nucleotide identities of the obtained sequences of bovine torovirus compared to bovine torovirus isolate (AY427798)

Sample IDs	Nucleotide identities (%)					
	Complete genome	pol1ab	S	M	HE	N
12151	80.3	78.9	95.5	94.3	87.1	69.8
37284	82.1	78.9	95.5	94.3	87.1	69.8

Table A14. The nucleotide identities of the obtained sequences of dsRNA1 segment of *Cryptosporidium parvum* virus 1 compared to other reported sequence (NC_038843)

Sample IDs	Nucleotide identities (%)
	dsRNA1 (RdRp)
00245	95.8
00562	95.6
18707	96.1
71346	95.8
85282	95.8
NA_4_516	96.1

Table A15. The nucleotide identities of the obtained sequences of dsRNA2 segment of *Cryptosporidium parvum* virus 1 compared to other reported sequence (NC_038844)

Sample IDs	Nucleotide identities (%)
	dsRNA2 (capsid protein)
00245	97.8
00562	98.1
18707	97.8
71346	97.8
85282	98.0
NA_4_516	98.0

Table A16. The nucleotide identities of the obtained sequences of hunnivirus compared to hunnivirus isolate (NC_018668)

Sample IDs	Nucleotide identities (%)											
	Complete genome	**L**	**VP4**	**VP2**	**VP3**	**VP1**	**2B**	**2C**	**3A**	**3B**	**3C**	**3D**
18897	81.3	87.3	84.8	65.4	69.1	57.1	85.6	87.5	82.6	84.0	86.5	90.1
23358	83.9	84.1	81.5	75.0	77.7	71.2	84.9	87.0	85.0	86.4	85.3	90.6
85282	81.2	87.3	83.1	65.7	70.4	59.6	82.1	88.1	84.4	85.2	86.5	90.3

국문초록

한우 송아지 설사에 대한 임상병리학적 변화 및 병원체 평가

채 정 병

(지도교수: 채 준 석)

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수의과대학 임상수의학 (수의내과학) 전공

송아지 설사는 다양한 원인에 의해 발병하는 질병으로 소화장기를 통해 수분의 소실을 일으키는 질병이다. 송아지 설사는 송아지의 성장을 저해하고 폐사까지 일으킬 수 있으며, 전세계의 소 산업에 큰 피해를 일으키는 질병이다. 많은 연구자들이 송아지 설사의 원인, 예방과 치료에 대한 연구를 지속하고 있음에도 불구하고, 아직 송아지 설사에 대한 근본적인 해결책을 찾지 못해 지속적으로 피해를 입고 있다. 따라서 본 연구는 국내 한우에서 발생하는 송아지 설사의 이해를 넓혀서 질병의 피해를 저감하고자 하였다.

우선, 첫 번째 연구는 한우 송아지에서 설사의 진단과 예후에 유용한 혈액검사항목에 대해 분석하였다. 국내 10개의 농장으로부터 530마리의 한우 송아지의 분변점수를 0점에서 3점(0점, 정상; 1점, 반고형; 2점, 묽은 변; 3점, 수양성 설사)까지 평가하고, 혈액을 확보하여 혈액검사, 혈청 생화학검사와 급성단백질을 검사하였다. 530마리의 한우 송아지 중 분변점수 0점, 1점, 2점, 3점은 각각 145마리, 185마리, 114마리, 88마리로 분류되었다. 모든 혈액검사결과들은 분변점수에 따라 통계적으로 비교하였을 때, 적혈구(red blood cells), 혈색소(hemoglobin), 평균혈구혈색소농도(mean corpuscular hemoglobin concentration), 망상적혈구(reticulocytes), 백혈구(white blood cells), 림프구(lymphocytes), 단핵구(monocytes), 알부민(albumin), 혈중 질소농도(blood urea nitrogen), 혈당(glucose), 혈중 나트륨농도(sodium), 혈중 칼륨농도(potassium), 섬유소원(fibrinogen), 그리고 합토글로빈(haptoglobin)이 분변점수에 따라 유의적인 변화를 나타내었다($p<0.01$). 그 후, 16개의 정상수치의 범위를 계산한 뒤, 정상수취 범위를 기준으로 각 분변 점수별 송아지 마리 수의 변화를 통계적으로 비교하였을 때, 혈액요소질소(blood urea nitrogen), 혈당(glucose), 혈중 나트륨농도(sodium), 혈중 칼륨농도(potassium), 섬유소원(fibrinogen), 그리고 합토글로빈(haptoglobin)에서 유의적인 차이를 나타내었다($p<0.001$). 6개의 혈액항목 중에서, 혈당과 혈중 나트륨농도는 분변점수가 증가함에 따라 기준치보다 낮은 송아지의 분포

가 유의적으로 증가한 반면($p<0.001$), 혈중질소농도, 혈중 칼륨농도, 섬유소원과 합토글로빈은 분변점수가 증가함에 따라 기준치보다 높은 송아지의 분포가 유의적으로 증가하였다($p<0.001$). 6개의 혈액검사 항목 중에서, 수신자 조작 특성 곡선(receiver operating characteristics curve, ROC curve) 아래 영역(area under ROC curve)이 0.5 이상이었던 혈중질소농도(9.50mg/dL), 혈중 칼륨농도(5.75mmol/L), 섬유소원(650.0mg/dL), 합토글로빈(12.5mg/dL)의 최적의 절사점이 계산되었다. 본 연구의 결과는 한우 송아지가 설사가 있을 때 숙주의 상태를 이해하는 데 큰 도움이 될 것으로 기대된다.

두 번째 연구에서는 한우 송아지에서 송아지 설사증을 일으킬 수 있는 병원체에 대해 조사하고, 농장, 계절, 분변점수와 병원체의 관계를 분석하였다. 2016년부터 2017년까지 국내 10개 농장으로부터 총 544개의 분변 샘플을 확보하여 7개의 감염병 원인체(소 로타바이러스(bovine rotavirus), 소 코로나바이러스(bovine coronavirus), 작은와포자충(*Cryptosporidium parvum*), 소 바이러스성 설사병 바이러스(bovine viral diarrhea virus), 구포자충속(*Eimeria* species), 대장균 K99 (*Escherichia coli* K99), 살모넬라 속(*Salmonella* species)들을 중합효소연쇄반응으로 조사하였다. 그 결과, 544건의 분변 중에서 분변 점수가 0점, 1점, 2점, 3점인 분변의 수는 각각 153건, 187건, 116건, 88건으로 조사되었다. 본 연구에서는 여름에 확보된 분변들의 점수가 다른 계절에 비해 유의적으로 높게 나타났으며($p<0.05$), 병원체

가 있는 분변 점수가 병원체가 없는 분변 점수보다 유의적으로 높게 나타났다($p<0.01$). 7개의 병원체 중에서, 6개의 병원체가 검출되었으며, 각 병원체별 검출율과 평균 분변점수는 다음과 같다: 구포자충속(27.4%, 1.36), 소 로타바이러스(8.8%, 1.85), 소 코로나바이러스(8.5%, 1.36), 작은와포자충(4.4%, 1.91), 소 바이러스성 설사병 바이러스(0.7%, 0.5), 대장균 K99 (0.2%, 3). 이 중에서 작은와포자충($p<0.01$)과 소 로타바이러스($p<0.001$)만 송아지의 분변점수가 증가함에 따라 유의적으로 높게 되었으며, 소 코로나바이러스는 작은와포자충($p<0.01$)과 소 로타바이러스($p<0.05$)와 동시에 감염되어 있을 확률이 유의적으로 높게 나타났다. 이러한 연구 결과는 한우 송아지 설사병의 숙주-병원체 상관관계와 역학을 이해하고 백신 개발 등 예방연구에 큰 도움이 될 것으로 기대된다.

세 번째 연구는 송아지 설사와 관련된 7가지 병원체(소 로타바이러스, 소 코로나바이러스, 소 바이러스성 설사바이러스 1, 2형, 작은와포자충, 편모충 속(*Giardia* species), 구포자충 속의 유병률을 중합효소연쇄반응으로 조사하고, 그 외 다른 바이러스들을 조사하기 위해 차세대염기서열분석 방법을 이용하였다. 2022년 한우 송아지 분변 총 810건을 채취하였고, 분변의 상태는 정상 분변 526건(분변 점수 0점 267건, 분변 점수 1점 259건)과 설사 분변 284건(분변 점수 2점 178건, 분변 점수 3점 106건)으

로 나누어졌다. 분변에서 중합효소연쇄반응으로 7가지 병원체 모두 검출되었으며, 각 병원체별 검출율과 평균 분변점수는 다음과 같다: 소 로타바이러스(14.0%, 1.41), 소 코로나바이러스(3.2%, 1.42), 바이러스성 설사병 바이러스 1형(2.1%, 1.35), 바이러스성 설사병 바이러스 1형 (4.9%, 1.33), 작은와포자충(9.8%, 1.66), 구포자충 속(1.9%, 1.73), 편모충 속(0.9%, 0.71). 그 중 소 로타바이러스($p<0.01$), 작은와포자충($p<0.001$), 구포자충 속($p<0.05$)만 분변점수가 증가함에 따라 검출율이 유의적으로 증가하였다. 분변 중 위의 병원체 7종에 대해 음성이었던 21건의 분변을 대상으로 송아지 설사와 관련된 바이러스 병원체를 확인하기 위해 차세대 염기서열 분석을 하였으며, 그 결과 소 코부파이러스(bovine kobuvirus), 부세피바이러스 B(boosepivirus B), 소 아스트로바이러스(bovine astrovirus), 소 파레코바이러스(bovine parechovirus), 소 토로바이러스(bovine torovirus), 작은와포자충 바이러스 1, 소 엔테로바이러스(bovine enterovirus), 소 네보바이러스(bovine nebovirus), 소 노로바이러스(bovine norovirus), 후니바이러스(hunni-virus)의 거의 완전한 유전자 서열을 확보하였다. 본 연구에서 부세피바이러스, 작은와포자충 바이러스 1과 후니바이러스의 존재와 10개의 신, 변종바이러스의 전체 유전자 서열을 국내 한우 송아지의 설사변에서 처음으로 검출하였다. 본 연구의 결과는 국내 송아지 설사와 관련된 병원체들의 역학과 분자생물학적 특징에 대한 이해도를 넓히는데 기여할 수

있을 것이다.

본 연구에서는 한우 설사 송아지의 전염성 병원체와 임상병리학적 변화에 대한 연구를 진행하였다. 본 연구는 송아지 설사의 임상수의학과 예방수의학의 관점을 접목하여 질병을 연구하였으며, 본 연구를 통해 우리는 한우 송아지에서 설사 질병의 이해를 깊게 하고, 이를 바탕으로 보다 효과적인 예방 및 치료 전략을 개발할 수 있을 것으로 기대한다. 추후 다양한 바이러스와 병원체들, 동시감염과 예방방법 등의 연구를 더 진행하여, 한우 송아지의 건강과 생산성을 증진시켜 축산농가의 경제적 손실을 줄이는데 기여할 것이다.

핵심어: 한우 송아지, 송아지 설사, 혈액학, 병원체, 역학조사, 신종 바이러스

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