



## RESEARCH ARTICLE

### Biological Factors Associated with Infectious Diarrhea in Calves

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#### ABSTRACT

Calf diarrhea is a major cause of economic loss in the cattle industry. Identifying biomarkers associated with the pathological conditions may provide beneficial guidelines for detecting disease progression and monitoring effectiveness of therapeutic agents in calves suffering from diarrhea. The objective of this research was to find out biological factors associated with calf diarrhea, based on clinical findings and the presence of enteric pathogens. Fecal and blood samples were obtained from 73 non-diarrheal and 42 diarrheal calves. The presence of enteric pathogens, hemato-chemical parameters, acute phase proteins, and pro-inflammatory cytokines were investigated in the samples. According to the presence of diarrhea and pathogens, the calves were classified into healthy (n=35), subclinical (non-diarrheal but pathogen-positive, n=36), infectious diarrheal (diarrheal and pathogen-positive, n=39), and unknown diarrheal groups (diarrheal but pathogen-negative, n=3). The presence of bovine coronavirus, bovine rotavirus group A, and *Cryptosporidium* spp. were significantly associated with the development of calf diarrhea ( $P<0.05$ ). The infectious diarrheal group showed increases in monocyte percentages and blood urea nitrogen level and the decreases in glucose, potassium, and phosphorus levels ( $P<0.05$ ). Moreover, inflammatory proteins such as haptoglobin, fibrinogen, interleukin-6, and tumor necrosis factor- $\alpha$  were elevated in the infectious diarrheal group compared to the healthy group ( $P<0.05$ ). The current study identified altered blood biological factors in calves with infectious diarrhea. The results suggested that these factors may be useful targets for monitoring animal health in calf diarrheal disease, especially infectious diarrhea.

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#### INTRODUCTION

Calf diarrhea is a common disease with high morbidity and mortality in young animals (Cho *et al.*, 2013). Multiple factors including a pathogen, environment, nutrition, and husbandry management affect the development of calf diarrhea (Al Mawly *et al.*, 2015). Due to the multifactorial nature of the disease, calf

diarrhea is regarded as a difficult disorder to effectively control (Cho and Yoon, 2014). In order to control complications of calf diarrhea, appropriate measures to alleviate the clinical symptoms should be taken concurrently with the identification of causal factors. The most important thing in the management of the disease is monitoring reliable markers that reflect pathological conditions in calves suffering from diarrhea, because it

takes considerable time to diagnose the cause of diarrhea (Blanchard, 2012; Huang *et al.*, 2020). Therefore, investigating biological alterations associated with calf diarrheal disease would be a key element in predicting disease progression and in treating the disease in a timely manner.

Some of variables including hematological, metabolic and immunological parameters can be used as biomarkers in response to disease (Marcato *et al.*, 2018). Blood hematological and biochemical indicators have been assessed to detect the cellular and metabolic changes in calves with diarrhea (Gultekin *et al.*, 2019). Moreover, several inflammatory proteins have been targeted as a biomarker to determine the prognosis of calf diarrhea (Chae *et al.*, 2019; Beheshtipour and Raeeszadeh, 2020). Specifically, acute phase proteins (APPs) are considered to be quantitative indicators of an innate immune response, which is correlated with the acuteness of infection, inflammation, or tissue injury (Hajimohammadi *et al.*, 2013). Haptoglobin (Hp), serum amyloid A (SAA) and fibrinogen (Fb) have been evaluated as major APPs in various infectious disease (Prohl *et al.*, 2015; Choi *et al.*, 2021), and as predictors of morbidity and mortality in calves (Seppa-Lassila *et al.*, 2015). The production of APPs is believed to be stimulated and regulated by cytokines such as interleukins (ILs), tumor necrosis factor-alpha (TNF- $\alpha$ ), and interferon-gamma (IFN- $\gamma$ ) (Ametaj *et al.*, 2011). A few studies have reported the production of pro-inflammatory cytokines in piglets with intestinal infection (Shi *et al.*, 2019) and neonatal calf diarrhea (Fischer *et al.*, 2016).

Despite efforts to find out diarrhea-related biomarkers, there is still a lack of information regarding the association among diarrhea, etiological factors, and pathophysiological parameters. Additionally, little is known about the inflammatory proteins and proinflammatory cytokines relating to the onset of diarrhea in calves; their changes during calf diarrhea remain to be investigated. Therefore, the aim of this study was to 1) identify potential bovine enteric pathogens related to the development of calf diarrhea, 2) investigate blood biological changes in calves based on diarrheal symptoms and enteric-pathogen infections, and 3) propose useful indicators related to different condition of calf diarrhea. This study would provide integrated information on calf diarrheal disease through the clinicopathological assessment and changes in physiological and immunological indices.

## MATERIALS AND METHODS

**Sample collection:** This study was conducted on nine farms in the Republic of Korea from April to October 2016. A total of 115 fecal and blood samples were collected from Korean Native (Hanwoo) or Holstein calves under the age of five months. The calves were clinically examined for health status and history of disease. Fecal samples were obtained from the rectum of each calf. Calves were divided into two groups according to a fecal consistency (Mcguirk, 2008): normal or semi-formed consistency was considered as non-diarrheal (n=73) while loose or watery feces as diarrheal (n=42). The blood samples were collected from the jugular veins.

All procedures were carried out according to ethical guidelines for the use of animal samples, as approved by Jeonbuk National University (Institutional Animal Care and Use Committee [IACUC] Decision No. CBU 2014-00026).

**Examination for intestinal pathogens:** Fecal samples were examined for enteric-pathogens including bovine coronavirus (BCoV), bovine rotavirus group A (BRV-A), bovine viral diarrhea virus (BVDV), *Cryptosporidium* spp., *Giardia* spp., *Eimeria* spp., *Escherichia coli* (*E. coli*) K99<sup>+</sup>, and *Salmonella* spp. The presence of pathogens was determined using real-time polymerase chain reaction as previously described (Cho *et al.*, 2010) or a Rapid BovID-5 Ag Test Kit (Bionote Inc., Korea) according to manufacturer's instructions except for the detection of *Eimeria* spp. The detection of *Eimeria* oocysts was done using the Sheather's sugar floatation procedure and microscopic examination (Rhee *et al.*, 1995). Fecal samples in which no pathogens were detected were considered to be pathogen negative. Based on the fecal consistency and pathogen detection, calves were subdivided into four groups healthy (n=35), subclinical (non-diarrheal but pathogen-positive, n=36), infectious diarrheal (diarrheal and pathogen-positive, n=39), and unknown diarrheal groups (diarrheal but pathogen-negative, n=3) to compare changes in hemato-chemical values, APP, and pro-inflammatory cytokines among groups.

**Hemato-chemical analysis:** Complete blood counts (CBCs) and serum chemistry were measured using a ProCyt Dx hematology analyzer (IDEXX Laboratories, Westbrook, MA, USA) and a Catalyst One™ chemistry analyzer (IDEXX Laboratories), respectively. The chemistry profile included total protein, albumin, blood urea nitrogen (BUN), glucose, sodium (Na), potassium (K), chloride (Cl) and phosphorus (P).

**Measurement of acute phase proteins and cytokines:** Enzyme-linked immunosorbent assay (ELISA) kits for Phase SAA Assay and Phase Hp Assay (Tridelata Development Ltd., Wicklow, Ireland) were used to determine serum SAA and Hp concentrations, respectively. Fb concentrations were calculated by subtracting the serum protein level from the plasma protein level as measured by a Reichert Vet 360 refractometer (Depew, NY, USA). Serum levels of TNF- $\alpha$  and IL-6 were measured with a Bovine TNF- $\alpha$  and IL-6 ELISA kit (R&D systems, Minneapolis, MN, USA).

**Statistical methods:** Statistical analysis was performed using IBM SPSS 23.0 (SPSS, Chicago, IL, USA). Binary logistic regression was applied to predict the odds of association between pathogen-detection and diarrhea-status. The association between the number of detected pathogens and diarrhea was determined using an ordinal regression model. Pearson's chi-square test ( $\chi^2$ ) was applied to determine specific pathogens related to the incidence of diarrhea. The odds ratio (OR) at a 95% confidence interval (CI) was calculated to assess the likelihood of association. CBC, serum chemistry, APP, and cytokine levels among groups were compared by either one-way analysis of variance or Kruskal-Wallis

test, depending on the distribution of normality. Data were expressed as the mean±standard deviation (SD) and P-values <0.05 were considered significant.

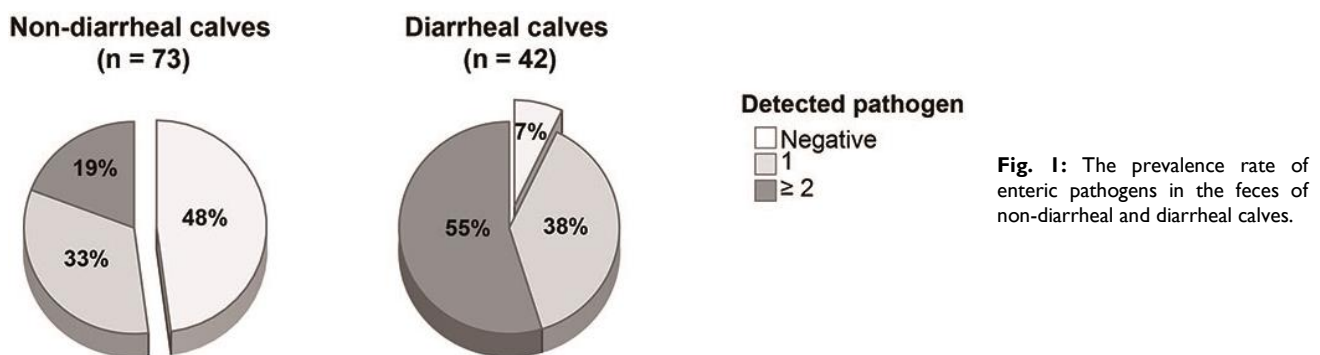
## RESULTS

**Enteric pathogens related to calf diarrhea:** Fecal samples were obtained from 115 calves, of which 42 calves (36.5%) were diagnosed with diarrhea (Fig. 1). At least one pathogen was detected in 93% of the fecal samples from diarrheal calves, and in 52% from non-diarrheal calves. The diarrheal calves were 11.97 times more likely to be infected with a pathogen than the non-diarrheal calves ( $P<0.001$ ,  $OR=11.97$ ,  $95\% CI=3.39-42.25$ ). In addition, multiple pathogens were observed in 55% and 19% of fecal samples from diarrheal calves and non-diarrheal calves, respectively. There was a correlation between the incidence of diarrhea and the increased number of pathogens detected, showing that diarrheal calves were 6.57 times more likely to be infected with more than two pathogens than non-diarrheal calves ( $P<0.001$ ,  $OR=6.57$ ,  $95\% CI=3.00-14.40$ ). Pathogen infections involving multiple pathogens were positively correlated with the incidence of diarrhea.

To determine which pathogens are related to calf diarrhea, the detection frequencies of eight bovine enteric

pathogens were investigated among diarrheal and non-diarrheal calves (Table 1). BCoV, BRV-A, *Cryptosporidium* spp., *Giardia* spp., *Eimeria* spp., and *E. coli* K99<sup>+</sup> were present in 36.5, 8.7, 13.9, 11.3, 35.7 and 4.3% of total fecal samples, respectively. Among them, BCoV was a major enteric pathogen associated with the incidence of diarrhea with strong odds ratio ( $P=0.000$ ,  $OR=10.30$ ,  $95\% CI=4.24-25.01$ ), followed by BRV-A ( $P=0.02$ ,  $OR=4.67$ ,  $95\% CI=1.14-19.15$ ) and *Cryptosporidium* spp. ( $P=0.02$ ,  $OR=3.49$ ,  $95\% CI=1.17-10.44$ ). *Giardia* spp., *Eimeria* spp., and *E. coli* K99<sup>+</sup> did not show the association with the incidence of diarrhea. No BVDV and *Salmonella* were detected from the samples.

When the comparison of disease incidence was done according to the age of the animals (Fig. 2), in calves over 31 days of age, BCoV was more frequently detected in diarrheal calves than in non-diarrheal calves ( $P<0.05$ ,  $OR=4.50$ ,  $95\% CI=0.62-32.70$ ), while in calves under 30 days of age, BRV-A was the predominant pathogen present in diarrheal calves compared to non-diarrheal calves ( $P<0.05$ ,  $OR=8.67$ ,  $95\% CI=0.87-86.06$ ). Additionally, *Cryptosporidium* spp. and *Eimeria* spp. were associated with diarrhea in animals 31–60 days of age ( $P<0.005$ ,  $OR=11.81$ ,  $95\% CI=2.04-68.55$ ) and 11–30 days of age ( $P<0.05$ ,  $OR=8.44$ ,  $95\% CI=0.82-86.83$ ), respectively.



**Fig. 1:** The prevalence rate of enteric pathogens in the feces of non-diarrheal and diarrheal calves.

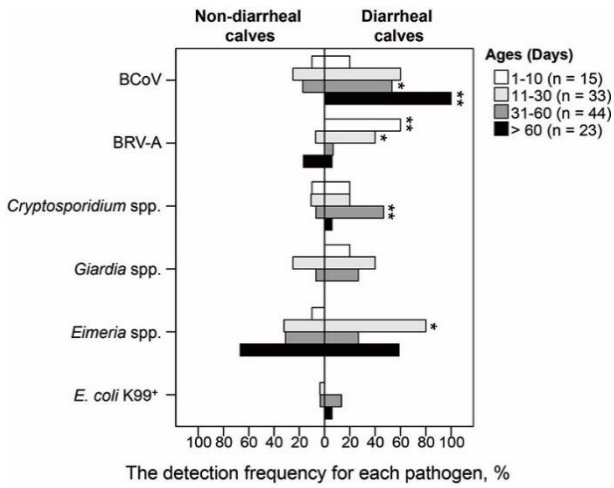
**Table 1:** Frequency of detection of each bovine enteric pathogen in the feces of diarrheal and non-diarrheal calves

| Pathogen                        | Overall positives (%) | Positives among diarrheal calves (%) | Positives among non-diarrheal calves (%) | P-Value | Odds ratio (95% CI) |
|---------------------------------|-----------------------|--------------------------------------|--|---------|---------------------|
| BCoV                            | 36.5                  | 69.0 (29/42)                         | 17.8 (13/73)                             | 0.00    | 10.30 (4.24–25.01)  |
| BRV-A                           | 8.7                   | 16.7 (7/42)                          | 4.1 (3/73)                               | 0.02    | 4.67 (1.14–19.15)   |
| <i>Cryptosporidium</i> spp.     | 13.9                  | 23.8 (10/42)                         | 8.2 (6/73)                               | 0.02    | 3.49 (1.17–10.44)   |
| <i>Giardia</i> spp.             | 11.3                  | 16.7 (7/42)                          | 8.2 (6/73)                               | 0.17    | 2.23 (0.70–7.16)    |
| <i>Eimeria</i> spp.             | 35.7                  | 42.9 (18/42)                         | 31.5 (23/73)                             | 0.22    | 1.63 (0.74–3.58)    |
| <i>E. coli</i> K99 <sup>+</sup> | 4.3                   | 7.1 (3/42)                           | 2.7 (2/73)                               | 0.35    | 2.73 (0.44–17.05)   |

**Table 2:** WBC and RBC profiles among calf groups categorized by diarrhea and detected pathogen

| Group     | WBC (K/ $\mu$ L) | NEU (%)   | LYM (%)   | MON (%)     | EOS (%)   | BAS (%)     |
|-----------|------------------|-----------|-----------|-------------|-----------|-------------|
|           | 4.0-12.0         | 15.0-47.0 | 45.0-75.0 | 0.0-11.0    | 0.0-20.0  | 0.0-3.5     |
| H (n=35)  | 10.2±3.4         | 34.9±12.7 | 52.8±11.1 | 10.7±5.0    | 1.4±2.0   | 0.1±0.2     |
| S (n=36)  | 10.3±3.5         | 36.1±11.1 | 51.5±11.6 | 11.2±3.7    | 0.9±1.5   | 0.3±0.5     |
| ID (n=39) | 10.9±3.5         | 28.1±15.5 | 51.5±14.0 | 19.5±12.0** | 0.7±1.8   | 0.1±0.2     |
| UD (n=3)  | 9.1±5.3          | 44.3±24.5 | 46.8±17.2 | 7.4±6.7     | 0.6±0.4   | 0.0±0.1     |
| Group     | RBC (M/ $\mu$ L) | Hb (g/dL) | HCT (%)   | MCV (fL)    | MCH (pg)  | MCHC (g/dL) |
|           | 5.0-10.0         | 8.0-15.0  | 24.0-46.0 | 40.0-60.0   | 11.1-17.0 | 28.2-36.0   |
| H (n=35)  | 10.3±2.4         | 10.2±2.3  | 32.5±7.5  | 32.3±6.5    | 10.2±3.2  | 31.8±10.2   |
| S (n=36)  | 10.8±1.5         | 11.1±2.7  | 34.7±8.1  | 32.3±6.1    | 10.3±2.2  | 31.9±3.7    |
| ID (n=39) | 9.9±2.2          | 10.6±2.7  | 32.4±7.7  | 34.1±7.5    | 11.0±2.5* | 32.7±4.0*   |
| UD (n=3)  | 8.4±1.6          | 7.9±2.2   | 24.0±3.1  | 29.4±6.3    | 9.8±3.5   | 32.5±5.7    |

H: Healthy (non-diarrheal and pathogen-negative), S: Subclinical (non-diarrheal, but pathogen-positive), ID: Infectious diarrheal (diarrheal and pathogen-positive), UD: Unknown diarrheal (diarrheal, but pathogen-negative), WBC: White blood cell, NEU: Neutrophil, LYM: Lymphocyte, MON: Monocyte, EOS: Eosinophil, BAS: Basophil, RBC: Red blood cell, Hb: Hemoglobin, HCT: Hematocrit, MCV: Mean cell volume, MCH: Mean cell hemoglobin, MCHC: Mean cell hemoglobin concentration. Mean±SD, \* $P<0.05$  and \*\* $P<0.01$  vs. Healthy.

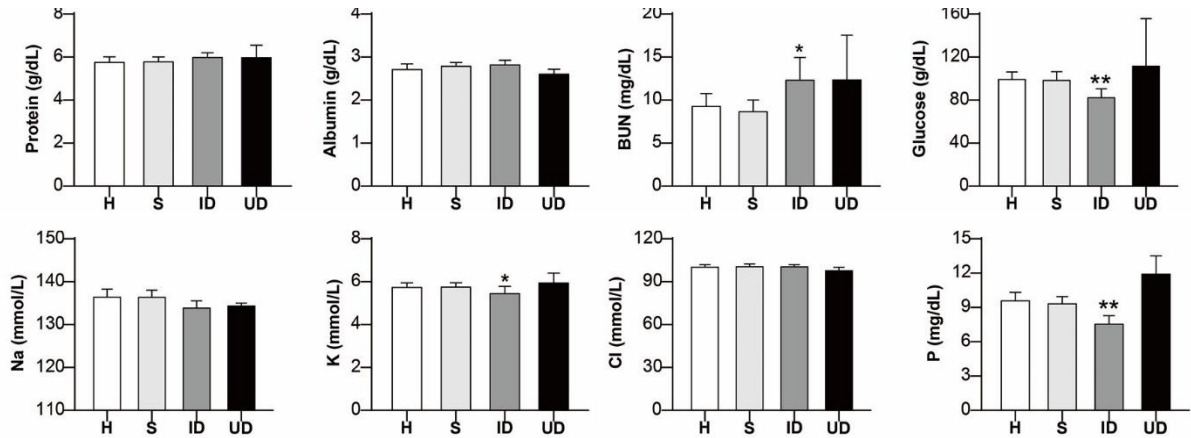


**Fig. 2:** Age-wise occurrence of enteric pathogens in diarrheal and non-diarrheal calves. \*P<0.05 and \*\*P<0.01 vs. Non-diarrheal calves.

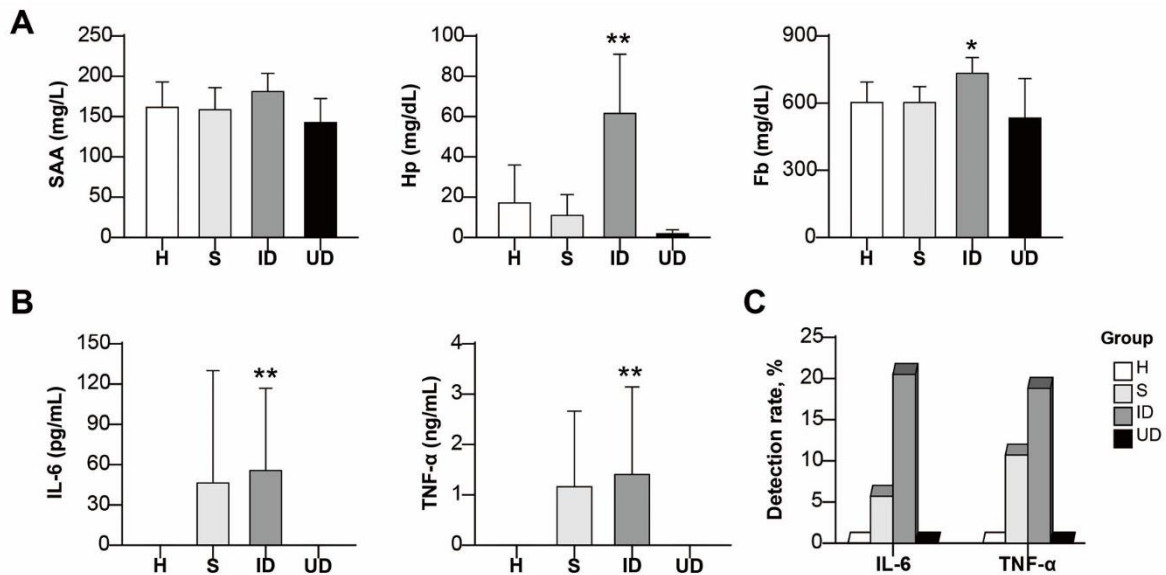
**Changes in hemato-chemical parameters relative to different diarrheal conditions:** Based on the analyses for the presence of diarrhea and pathogens, the 115 calves

were divided into four groups: the healthy group (non-diarrheal and pathogen negative), which was used as a control group; the subclinical group (non-diarrheal but pathogen positive); the infectious diarrheal group (diarrheal and pathogen positive); and the unknown diarrheal group (diarrheal, but pathogen negative). CBC and serum chemistry results were compared among the four groups.

There was no significant difference in total white blood cell (WBC) counts between groups, but the increased proportion of monocytes was observed in the infectious diarrheal group compared to the healthy group (P<0.01; Table 2). When the chemistry profile of each group was compared, there were no significant differences in the levels of total protein, albumin, Na, and Cl between groups. (Fig. 3). However, the infectious diarrheal group showed significantly higher level of BUN and lower levels of glucose, K, and P than the healthy group (P<0.05; Fig. 3). The results revealed that the monocyte, BUN, glucose, K, and P were physiological factors that can be perturbed in calves with infectious diarrheal condition.



**Fig. 3:** Comparison of serum biochemical parameters among different diarrheal conditions. H: Healthy, S: Subclinical, ID: Infectious diarrheal, UD: Unknown diarrheal groups, BUN: Blood urea nitrogen, Na: Sodium, K: Potassium, Cl: Chloride, P: Phosphorus. \*P<0.05 and \*\*P<0.01 vs. Healthy.



**Fig. 4:** Acute phase protein (APP) concentrations and cytokine levels among different diarrheal conditions. A) The concentrations of APP and fibrinogen (Fb). B) The concentrations of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α). C) Detection frequency of IL-6 and TNF-α. The concentrations of haptoglobin (Hp), Fb, IL-6, and TNF-α were elevated in infectious diarrheal (ID) group. H: Healthy, S: Subclinical, UD: Unknown diarrheal groups, serum amyloid A (SAA). \*P<0.05 and \*\*P<0.01 vs. Healthy.

**Immunological responses in different diarrheal conditions:** Serum concentrations of Hp and Fb were elevated in the infectious diarrheal group ( $P < 0.05$ ), while SAA level showed no significant difference among the groups (Fig. 4A). IL-6 and TNF- $\alpha$  concentrations were also elevated in the infectious diarrheal group compared to the healthy group ( $P < 0.01$ ; Fig. 4B).

IL-6 and TNF- $\alpha$  were found to be released under conditions of calves being infected with enteric pathogens (Fig. 4C). No IL-6 or TNF- $\alpha$  were detected in any of the healthy and the unknown diarrheal calves negative for the eight pathogens investigated in this study. Detection of these cytokines was only observed in the subclinical and the infectious diarrheal calves positive for at least one pathogen. Specifically, IL-6 was observed in 5.7% of the subclinical group and 20.5% of the infectious diarrheal group. TNF- $\alpha$  was observed in 10.7% of the subclinical group and 18.8% of the infectious diarrheal group. This finding indicated that inflammatory factors such as Hp, Fb, IL-6, and TNF- $\alpha$  were positively associated with diarrheal condition accompanied by enteric pathogen infection in calves.

## DISCUSSION

This is one of the few studies that explore pathological disturbances and propose potential biomarkers associated with calf diarrhea. Our findings provide fundamental information for useful biological indicators in assessing diarrheal status. Notably, this study identified distinct biological factors in diarrheal conditions with infectious agents. More than 90% of the diarrheal calves in our study were positive for enteric pathogens, indicating that infection was a major cause of calf diarrhea, as reported in the previous paper (Cho *et al.*, 2013). Therefore, studying the specific biological changes that occur in infectious diarrhea will provide important clues for understanding and treating the pathological condition of calf diarrhea.

Various enteric pathogens can be detected both in diarrheal and non-diarrheal calves, demonstrating a common frequency, even in the non-diarrheal samples (Seppa-Lassila *et al.*, 2015). The current study also showed that more than half (53%) of the fecal samples from non-diarrheal calves were positive for pathogens, although the pathogens were detected at a lower frequency in the feces of non-diarrheal calves than in the feces of diarrheal calves. The presence of pathogen itself may not correlate with the etiology of diarrhea (Seppa-Lassila *et al.*, 2015). The quantity of fecal shedding of the pathogens may play an important role in the development of diarrhea, as well as the frequency of detection of the pathogens (Cho *et al.*, 2013). A quantitative investigation would be useful for determining the clinical significance of pathogens. In addition, the pathogenic species or type of each pathogen should also be identified in future studies.

Consistent with our findings, BCoV, BRV-A, and *Cryptosporidium* spp. have been considered enteropathogens associated with calf diarrhea (Boileau and Kapil, 2010; Cho *et al.*, 2013). The parasite *Eimeria* spp. has been reported to be the relevant cause of calf diarrhea (Tamrat *et al.*, 2020), which begins to excrete oocyst in

calves around 3 weeks of age (Svensson, 1993). In agreement with the previous reports, the present investigation revealed that *Eimeria* spp. was closely associated with diarrhea at specific ages of calves, showing a significantly higher frequency of infection in diarrheal calves than non-diarrheal calves at 11-30 days of age.

Only three of total 115 calves showed diarrhea negative for pathogens. Non-infectious factors such as weather, or poor hygiene, nutrition or, animal care may have contributed to the development of calf diarrhea in these animals. However, the possibility of other pathogens that we did not test for this study remains to be further studied, which may include unrecognized or newly emerging agents involved in calf diarrhea.

Azotemia may occur during endogenous protein catabolism, probably due to tissue damage, intestinal hemorrhage, or dehydration (Steiger Burgos *et al.*, 2001). Others have shown that hematocrit, total protein, and BUN were prognostic factor associated with survival of calves with diarrhea (Seifi *et al.*, 2006). Elevated BUN levels were also reported previously in calves experimentally infected with a high dose of *E. zuernii* oocysts (Bangoura *et al.*, 2007). Hypoglycemia was found to be associated to septic neonatal calf diarrhea (Trefz *et al.*, 2016). In current study, increased BUN and decreased glucose concentrations were only observed in infectious diarrheal calves. BUN and glucose may be useful biochemical indicators reflecting metabolic alterations in clinical diarrhea with enteric pathogens. However, BUN and glucose concentrations may be easily affected by environmental factors or disease duration (Marcato *et al.*, 2018). Careful caution is required to determine the BUN and glucose as biomarkers of calf diarrhea. Additional research on them should be conducted under several conditions of infectious diarrhea (*e.g.* acute, chronic, recovery, or poor prognostic stages).

In general, diarrhea leads to an electrolyte imbalance and dehydration that is attributed to an elevated fecal-loss of electrolytes and increased intestinal-loss of water and nutrients (Bangoura and Dausgies, 2007). Typically, hyponatremia and hypo- or hyperkalemia occur in diarrheal calves based on the chronic or acute stage of the condition (Sayers *et al.*, 2016). Potassium is lost in feces and urine during diarrhea but it is also elevated due to K shifts from the intracellular fluid into extracellular fluid in response to metabolic acidosis (Seifi *et al.*, 2006). In the current study, no distinct imbalance following the development of diarrhea could be detected for Na or Cl concentrations among the groups, while decreases in K and P concentrations were seen in the infectious diarrheal group compared to the other groups. As far as we know, there is no report of P concentration in diarrheal calves, but the decrease in P concentration seems to be due to malabsorption or the loss of nutrients via the inflamed intestine induced by the infectious agents (Moe, 2008). Distinct change in P level appear to be one available biomarker for monitoring the recovery status of infectious diarrheal calves.

SAA and Hp, two major serum APPs in bovine, have been used to evaluate inflammatory conditions in cattle (Balikci and Al, 2014). In particular, Hp has been shown to have a higher sensitivity in detecting disease due to its

pronounced and prolonged response to infection (Angen *et al.*, 2009). Hp can be a useful indicator of bovine bacterial infections (Eckersall and Bell, 2010) or may be a potential disease prognostic factor (Choi *et al.*, 2021). In current study, serum Hp was also sensitive APP in detecting calf diarrheal disease caused by bovine enteric pathogens. The Hp concentration of the infectious diarrheal calves (61.5 mg/dL) was significantly higher than those of the healthy (17.1 mg/dL), the subclinical (10.9 mg/dL), and the unknown diarrheal (18.3 mg/dL) calves. This study suggests that serum Hp may be a better biomarker for infectious diarrheal disease in calves than other APPs. Along with these findings, further studies are needed to determine a value of APPs in predicting morbidity and mortality in calf diarrheal disease.

It was difficult to conclusively determine any association between enteric pathogens and cytokine production due to the low sensitivity of cytokine detection and the limited sample size of the unknown (non-infectious) diarrheal group in this study. A previous report described that IL-6 concentration was higher in non-recovered calves with diarrheal disease than recovered calves (Fischer *et al.*, 2016). Cytokine values may depend on the recovery time after the onset of diarrheal disease. Low levels of pro-inflammatory cytokines may be associated with the recovery process from diarrheal disease. Pro-inflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$  have also been reported to significantly increase in neonatal calf diarrheal disease (Beheshtipour and Raeeszadeh, 2020). The study suggested pro-inflammatory cytokines as a marker for recognizing the immune response of neonatal calf diarrheal disease. In the current study, elevated TNF- $\alpha$  and IL-6 were only observed in subclinical and diarrheal calves positive for enteric pathogens. Therefore, cytokine detection may be useful as an additional tool for estimating the causative factors of calf diarrheal disease, i.e., infectious contributors.

The increased relative percentage of monocytes in the peripheral blood may also indicate a diarrheal condition being infectious in nature. Monocytes appear to mediate innate immunity and to actively initiate adaptive immune responses in hosts infected with enteric pathogens (Taubert *et al.*, 2009). Monocytes in infected calves serve as precursors of macrophages and are considered to be associated with the release of TNF- $\alpha$  and IL-6, and with the production of APP induced by the released cytokines (Taubert *et al.*, 2009; Ametaj *et al.*, 2011). Similar to previous study indicating that monocytes were correlated with diarrheal disease with BCoV in post-weaned calves (Chae *et al.*, 2019), the increased percentage of monocytes was observed in infectious diarrheal group in the present study. Increased monocytes appear to be closely associated with multiple results of infection and diarrheal disease. Consequently, the production of APP may have been induced by the pro-inflammatory cytokines, which were secreted from increased monocytes in infectious diarrheal disease. Finally, serum Hp, in combination with monocytes and cytokine levels, can be promising indicators for infectious diarrheal disease in calves. In general, this study highlights potential biological candidates for evaluating the health of calves suffering from diarrheal disease and managing their diarrheal disease.

**Conclusions:** Infection was a major risk factor for calf diarrheal disease, and co-infection with multiple pathogens was common. Infectious diarrheal disease led to hematological and biochemical alterations, and an excessive immune response. This included changes in monocytes, BUN, glucose, K, P, Hp, IL-6, and TNF- $\alpha$ . The screening of these parameters will be helpful for the therapeutic intervention and subsequent monitoring of infectious diarrheal disease in calves. This study highlights biological candidates for evaluating and treating infectious diarrheal disease as a significant cause of calf diarrheal disease.

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**Authors contributions:** DHY, KSC and JP designed the study. SK and JP drafted the manuscript. SJ, JK, KP and JBC collected samples. SJ, JK, KSC, HCK and BKP performed analysis. KSC and JSC provided consultation. All authors read and approved the final manuscript.

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