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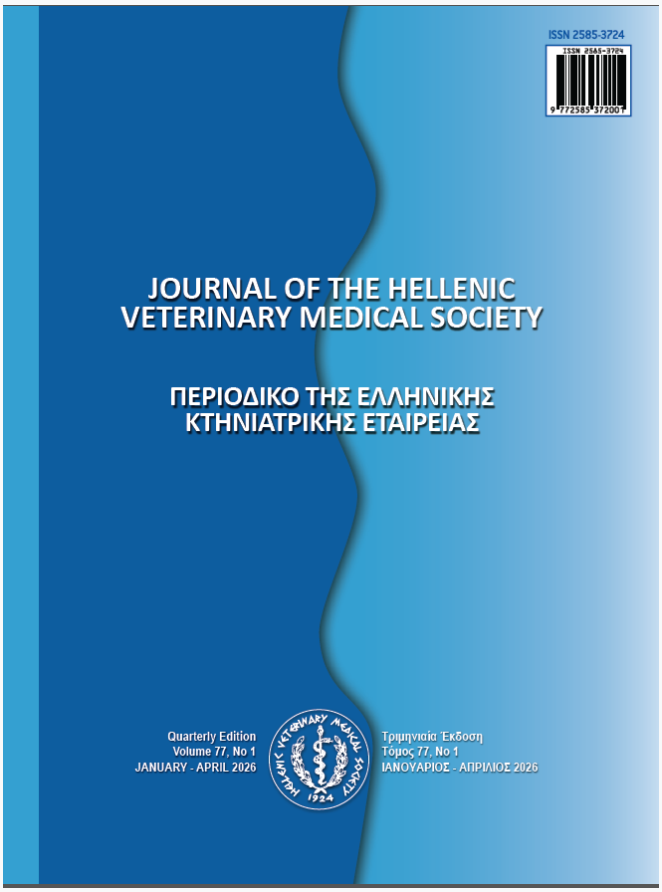

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Research article
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Cardiac Biomarkers in Cryptosporidium-Induced Diarrhea: Evaluating Sepsis-Associated Myocardial Dysfunction in Calves

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ABSTRACT: The aim was to investigate the relationship between cardiovascular damage and sepsis in calves infected with *Cryptosporidium* spp. and to evaluate the effectiveness of cTnI and NT-proBNP as biomarkers. 21 calves were selected from 133 calves under the inclusion/exclusion criteria. Diarrheic calves with (n=7) and without sepsis (n=7) all affected by *Cryptosporidium* spp., and clinically healthy calves (n=7) were studied. The diagnostic efficacies of NT-proBNP and cTnI concentrations were evaluated across three groups. cTnI concentration exhibited significant differences among all the groups ($p<0.001$), with the highest level observed in the Sepsis group (256.13±88.23 pg/mL). The serum NT-proBNP concentration was highest in the sepsis group (162.12 ± 117.52 pg/mL). A statistically significant difference was observed between the sepsis and healthy groups (25.58 ± 13.24 pg/mL), as well as between the non-sepsis (112.39 ± 54.99 pg/mL) and healthy groups. These findings suggest the possibility of cardiovascular disorders in neonatal diarrhea caused by *Cryptosporidium* spp., even without sepsis, indicating that the parasite may contribute to systemic effects. Cardiac assessment should be considered during diagnosis and treatment of neonatal calf diarrhea, even in the absence of sepsis.

Keyword: Calf; Cardiovascular disorder; Cryptosporidiosis; Enteritis; Neonatal

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INTRODUCTION

The neonatal period, spanning the first 28 days of a calf's life, is a critical phase during which physiological functions adjust to postnatal life (Constable et al., 2017). Neonatal diarrhea, the primary infectious problem in calves, is followed by pneumonia in older neonatal calves aged 2-28 days or even older leading to significant neonatal morbidity and mortality, which in turn causes substantial economic losses (Nagy, 2009).

Neonatal calf diarrhea can result from infectious and non-infectious factors, including management, nutrition, and environmental conditions. "While multifactorial, key infectious causes involve bacteria such as *Clostridium perfringens* and *Escherichia coli*; viruses like *Coronavirus* and *Rotavirus*; and parasites, among which *Cryptosporidium* spp. is recognized as the leading cause of enteric disease in calves under one month of age. In fact, it accounted for 37% of diagnosable cases in a 5-year veterinary surveillance report (Blanchard, 2012; Hotchkiss et al., 2015).The re-emerging role of *Cryptosporidium* spp. is supported by a growing body of anecdotal evidence that can no longer be overlooked (Fayer, 2010; Hotchkiss et al., 2015).

Sepsis, a life-threatening condition caused by the body's dysregulated response to infectious pathogens or their toxins in the bloodstream, is a leading cause of mortality in both animals and humans. Sepsis in calves was attributed to intestinal mucosal damage caused by bacterial, viral, or parasitic gastrointestinal infections, which allowed opportunistic gut pathogens to enter the systemic circulation (Fecteau et al., 1997; Pas et al., 2023). Studies in neonatal calves with sepsis suggest that impaired cellular metabolism and circulatory dysfunction, not systolic dysfunction, are the primary clinical abnormalities in septic neonatal calves (Naseri et al., 2019). Cardiac troponin I (cTn-I) and N-terminal prohormone of brain natriuretic peptide (NT-proBNP) play a crucial role in early cardiac disorder detection (Aygun and Yildiz, 2018). Previous studies explored these cardiovascular biomarkers in calves with neonatal diarrhea linked to *Salmonella* spp. and *E. Coli* (Shehta et al., 2022).

Although *Cryptosporidium* spp. is a well-known cause of neonatal diarrhea in calves, data on its cardiac effects are limited. To our knowledge, no study has evaluated cardiac dysfunction in calves with *Cryptosporidium*-associated diarrhea while distinguishing between septic and non-septic cases. We hypothesized that cardiac dysfunction occurs in calves with Cryptosporidium-induced diarrhea regardless of sepsis status. This study addresses that gap by investigating cardiac biomarker changes in both septic and non-septic calves infected with *Cryptosporidium* spp., offering new insights into the protozoan cardiac-specific effects of this emerging zoonotic pathogen.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the Harran University Animal Experiments Local Ethics Committee (28.03.2022, 2022/002 – 01-13 Number Ethics Committee Decision).

Animals

A total of 133 calves presented to the Animal Hospital of Harran University Faculty of Veterinary Medicine for diagnosis, treatment, and/or routine health assessment were initially considered for this study. Based on predefined inclusion and exclusion criteria and due to the low incidence of monoinfection, 21 neonatal calves were ultimately selected for inclusion. Among these, 9 were Holstein and 12 were Simmental breeds; 9 were male and 12 were female. Upon hospital admission, a detailed history was obtained from the farmer/producer, including the types and duration of symptoms. Concurrently, a clinical examination was performed, and the necessary samples were collected.

Physical Examinations, Collection of Blood and Fecal Samples

Within the scope of the physical examination of all the calves deemed appropriate to be included in the study, lung and heart auscultation, palpable lymph node and mucous membrane evaluations, pulse, respiratory rate and rectal body temperature measurements were performed. Also, calf health score (CHS) was calculated at the penside for each calf (McGuirk et al., 2008). Moreover, the degree of dehydration of each calf was determined based on skin tent duration and eyeball recession (Smith, 2009). Venous blood samples were taken from all the calves via vena jugularis venipuncture following appropriate asepsis and antisepsis protocol (5-7 mL). A portion of the blood samples (1-3 mL) were transferred to K3EDTA tubes and used for complete blood count (CBC) (using an autoanalyzer; Sysmex pocH 100i, Japan). The remaining blood sample (3–4 mL) was transferred into gel-containing tubes for serum separation and

subsequent biomarker analysis. Additionally, fecal samples were directly collected from the rectum of all calves into sterile plastic containers for further examination. According to the anamnesis provided by the animal owners at hospital admission, the clinical symptoms in calves had typically been present for 1 to 3 days prior to sample collection.

Diagnosis of *Cryptosporidium* spp. Infection

Gross and microscopic examination of all fecal samples were performed using light microscopy (x40 magnification, Olympus, Japan). Detection of the *Cryptosporidium* spp. oocyst by using modified Ziehl Nielsen (MZN) stain (EDM, CAT. NO. 2995) was performed according to previously reported method (Fayer and Xiae, 2008). In addition, the diagnosis of *Cryptosporidium* spp. was confirmed with immunochromatographic rapid diagnostic test kits (BIO K 306 - Rainbow Calf Scours 5, Belgium) in fecal samples.

Determining The Presence of Sepsis

Following the initial clinical examination, sepsis and systemic inflammatory response syndrome (SIRS) criteria were assessed based on the presence of depression, reduced or absent suckling reflex, diarrhea, persistent recumbency, and dehydration. According to this examination, SIRS criteria for neonatal calf as follows; rectal body temperature >39.5 °C or <37 °C, respiratory rate >45 per minute, heart rate <100 or >160 per minute, leukocyte count >12×103/μL or <4×103/μL (Sen and Constable, 2013; Yıldız et al., 2018). The presence of at least two of the specified criteria with suspicion or presence of infection was considered as sepsis and included in the present study (Lofstedt et al., 1999; Fecteau et al., 2009; Ayvazoğlu et al., 2024).

Inclusion/Exclusion Criteria

Due to its simplicity, rapidity, sensitivity and specificity, lateral immunochromatography-based strip test (BIO K 306 - Rainbow Calf Scours 5, Belgium) was used to investigate the etiological agents of diarrhea including *Cl. perfringens*, *Cryptosporidium* spp., *Coronavirus*, *Rotavirus* and *E. coli* F5. Calves testing positive for any viral, bacterial, or protozoal agents other than *Cryptosporidium* spp. using rapid diagnostic kits, as well as those with parasites and/or eggs/oocysts detected during microscopic examination (except for a positive MZN test for *Cryptosporidium* oocysts), were excluded from the study. Calves with congenital abnormalities, asphyxia and that received/receiving any treatment were also excluded from the study. Additionally, calves diagnosed with respiratory, neurological (including apathy and decubitus), and umbilical infections during clinical examination were excluded from the study. Therefore, only calves that developed diarrhea from *Cryptosporidium* spp., confirmed by rapid diagnostic test kits, were included and subsequently grouped based on the presence of sepsis.

Forming Subgroups

As a result of examinations, the following assignments were made: calves with diarrhea caused by *Cryptosporidium* spp. but without sepsis were assigned to the Non-sepsis group (n: 7), calves with diarrhea caused by *Cryptosporidium* spp. and sepsis were assigned to the Sepsis group (n: 7), and calves deemed healthy after all examinations were included in the Healthy group (n: 7). Although the power was below the optimal 80% threshold, the sample size per group was constrained to seven calves due to ethical and logistical reasons. However, a post hoc power analysis based on the observed data showed that this number provided moderate statistical power, especially for detecting differences in cTnI levels. Therefore, the sample size was considered sufficient for the scope of this exploratory study.

Biomarker Measurements

Venous blood samples in tubes for serum separation with gel were centrifuged at 2500 g for 10 minutes, serum samples were extracted and transferred to Eppendorf tubes and kept in the freezer at -80 °C for approximately 5 months until the biomarker measurement day. Although the storage period in our study was approximately five months at −80 °C, previous studies have demonstrated that both cardiac troponins and natriuretic peptites remain stable under such conditions for significantly longer durations (Basit et al., 2007; Cauliez et al., 2008; Lee and Aw, 2023). Therefore, we believe that the integrity of the biomarkers was well preserved and that the storage conditions are unlikely to have affected our results. cTnI and NT-proBNP concentrations were measured from the serum samples to investigate cardiovascular involvement in accordance with the manufacturer's instructions (Bovine cTn-I ELISA Kit and Bovine NT-proBNP ELISA Kit, Sandwich ELISA, Double Antibody, Wuhan Fine Biotech, China).

Statistical Analysis

All statistical analyses were performed using SPSS version 27 (IBM Corp., Armonk, NY, USA). The Shapiro-Wilk test was applied to assess the normality

of data distribution, given the small sample size (n = 7 per group Parametric data were presented as mean ± standard error of mean (SEM) and non-parametric data were presented as median and range (min-max). Although the Shapiro–Wilk test confirmed normal distribution, the median (IQR; Q1–Q3) values were provided along with the mean+SEM due to the small sample size and to provide more robust summary statistics. Data analysis of the three groups was performed with one-way ANOVA or Kruskal-Wallis test depending on the distribution pattern of data. When a statistically significant difference was identified, Dunn's post hoc test with Bonferroni correction was applied for non-parametric data, and Tukey's HSD test was used for parametric data to adjust for multiple comparisons. Receiver operator characteristic (ROC) analysis, with the area under the curve (AUC), was also performed to examine the diagnostic efficacies of serum cTnI and NT-proBNP concentrations. Optimal cut-off values were selected based on the lowest number of misclassifications, with sensitivity and specificity calculated accordingly. In the ROC analysis, an AUC of 0.5 indicates no discrimination, 0.6–0.8 is acceptable, 0.8–0.9 excellent, and >0.9 outstanding (Hosmer and Lemeshow, 2000). A p-value < 0.05 was considered statistically significant in all analyses.

To support the adequacy of this sample size, an a priori power analysis was conducted using G*Power software (Version 3.1). Assuming a large effect size (d = 1.5), an alpha level of 0.05, and statistical power (1–β) of 0.80 for a two-tailed t-test, the minimum required sample size was calculated to be six animals per group. Accordingly, the inclusion of seven animals per group was sufficient to detect large effect sizes with acceptable statistical power.

RESULTS

The demographic data and clinical history-based symptom durations of the cases in the groups were found to be similar to each other. Based on clinical history information taken when calves are brought to our hospital for treatment, symptom duration was 7 (4-10) days in the Sepsis group and 8 (4-10) days in the Non-sepsis group (P<0.85). Physical examinations revealed that the pulse rate was higher in all the diarrheic calves than in the healthy calves, regardless of the presence of sepsis (P<0.005). While CHS was not statistically different between the Sepsis and Non-sepsis groups, it was higher in diarrheic calves than in the healthy calves (P<0.001). Although the dehydration degree was statistically insignificant among the diarrheic calves, it was higher than the healthy ones (P<0.005). All physical examination, CHS and dehydration degree evaluation findings, analyzed non-parametrically, are presented in Table 1.

Within the scope of biomarker analysis, cTnI concentration differed among all the groups and was highest in the Sepsis group (P<0.001). Serum NT-proBNP concentration was statistically insignificant in the comparison of Sepsis and Non-sepsis groups but was higher than in the Healthy group (P<0.05). Biomarkers concentration measurement

Table 1. The statistical results of the clinical parameters in study groups

Parameters	Sepsis Group n:7 Median (min-max)	Non-Sepsis Group n:7 Median (min-max)	Healthy Group n:7 Median (min-max)	*p* value
Pulse rate (bpm)	128 (80-156)[a]	120 (90-136)[a]	88 (80-98)[b]	0.003
Respiration rate (brpm)	48 (28-60)	40 (22-58)	35 (19-59)	0.404
Body temperature (°C)	38.1 (36.2-39.6)	38 (36-39.3)	38.7 (38.2-39.3)	0.241
CHS	5 (3-7)[a]	4 (3-9)[a]	1 (0-2)[b]	0.000
Dehydration degree (%)	8 (6-10)[a]	8 (6-10)[a]	6 (5-8)[b]	0.001

*a, b: Letters indicate differences between groups at the chosen significance level (Dunn's test, p < 0.05). CHS: Calf health score, bpm: beat per minute, brpm: breath per minute

Table 2. The statistical results of cardiac disorder biomarkers in the research groups

Parameters	Sepsis Group N:7 mean±SEM	Non-Sepsis Group N:7 mean±SEM	Healthy Group N:7 mean±SEM	*P* value
cTnI (pg/mL)	256.13±88.23[a] (165.44 – 368.66)*	107.74±40.85[b] (74.04 – 143.66)*	43.39±14.35[c] (33.1 – 60.91)*	0.000
NT-proBNP (pg/mL)	162.12±117.52[a] (60.35 – 320.98)*	112.39±54.99[a] (49.76 – 159.52)*	25.58±13.24[b] (13 – 34.16)*	0.017

a, b: Letters indicate differences between groups at the chosen significance level (Tukey's HSD, $p < 0.05$). SEM: The standard error of the mean. *Median [Interquartile range (IQR; Q1 – Q3)]

results were analyzed parametrically and intergroup comparison are presented in Table 2.

As a result of the comparative ROC analysis, which was performed to distinguish the calves with sepsis from the healthy calves based on cardiac biomarkers, the diagnostic performances of cTnI and NT-proBNP were determined to be outstanding at cut-off values of 103.11 and 36.85, respectively (AUC=1.000 and AUC=0.971). ROC analysis comparison of Sepsis and Healthy groups is presented in Table 3.

As a result of the ROC analysis performed to distinguish the presence of sepsis in calves with neonatal diarrhea due to *Cryptosporidium* spp. based on cardiac biomarkers, it was determined that the serum cTnI concentration had an outstanding diagnostic performance at the cut-off value of 130.74 (AUC=0.943). Serum NT-proBNP concentration suggests no discrimination in determining the presence of sepsis in calves with diarrhea (AUC <0.600). The diagnostic performances of serum cTnI and NT-proBNP concentrations in distinguishing those with sepsis from those without sepsis (Sepsis vs Non-sepsis) are presented in Table 4.

Serum cTnI and NT-proBNP concentrations demonstrated outstanding diagnostic performance in distinguishing non-septic calves with neonatal diarrhea due to *Cryptosporidium* spp. from healthy calves, with cut-off values of 37.77 and 24.39, respectively (AUC = 0.918). The diagnostic efficacy of these biomarkers for differentiating Non-sepsis and Healthy groups is summarized in Table 5.

Table 3. Diagnostic performance of cTnI and NT-proBNP in distinguishing the calves with sepsis from the healthy ones

Parameter	AUC	Std. error	Asymp. Sig.	Asymp. 95% CI		Cut-off	Sensitivity	Specificity
				Lower Bound	Upper Bound			
cTnI (pg/mL)	1.000*	0.000	0.004	1.000	1.000	103.11	%100	%100
NT-proBNP (pg/mL)	0.971**	0.044	0.007	0.886	1.000	36.85	%100	%85.7

Std. error: Standard error, Asymp. Sig.: Asymptotic Significance, CI: Confidence interval. *(95% CI: 0.98–1.00). **(95% CI: 0.92–1.00).

Table 4. Diagnostic performances of serum cTnI and NT-proBNP concentrations in distinguishing those with sepsis from those without sepsis

Parameter	AUC	Std. error	Asymp. Sig	Asymp. 95% CI		Cut-off	Sensitivity	Specificity
				Lower Bound	Upper Bound			
cTnI (pg/mL)	0.943*	0.069	0.012	0.808	1.000	130.74	%100	%71.4
NT-proBNP (pg/mL)	0.543**	0.194	0.808	0.163	0.923	65.46	%80	%29.6

Std. error: Standard error, Asymp. Sig.: Asymptotic Significance, CI: Confidence interval. *(95% CI: 0.89–0.99). **(95% CI: 95% CI: 0.45–0.64).

Table 5. Diagnostic performances of serum cTnI and NT-proBNP concentrations in distinguishing non-septic calves from healthy ones

Parameter	AUC	Std. error	Asymp. Sig	Asymp. 95% CI		Cut-off	Sensitivity	Specificity
				Lower Bound	Upper Bound			
cTnI (pg/mL)	0.918*	0.084	0.009	0.754	1.000	37.77	%85.7	%42.9
NT-proBNP (pg/mL)	0.918**	0.080	0.008	0.754	1.000	24.39	%100	%42.9

Std. error: Standard error, Asymp. Sig.: Asymptotic Significance, CI: Confidence interval. *(95% CI: 0.86–0.97). **(95% CI: 95% CI: 0.86–0.97).

DISCUSSION

Diarrhea and various gastrointestinal tract disorders are the leading causes of calf mortality globally (Zhang et al., 2019). The significance of calf health and well-being has been emphasized in recent years, considering both short- and long-term perspectives (Lorenz, 2021). Calves experiencing diarrhea, as well as those who have recovered from it, release infectious agents into their surroundings, establishing them as primary sources of contamination. Consequently, the prompt identification and efficient treatment of diarrheic calves become crucial. It is worth noting that various pathogenic organisms contribute to the development of severe diarrhea in neonatal calves. Among these pathogens, protozoa like *Cryptosporidium* spp. represent one of the most significant infectious agents that can affect young calves and lead to mortality (Cho and Yoon, 2014).

In a study conducted on 10 calves with endotoxemia, it was reported that cTnI is a highly specific indicator for detecting myocardial damage (Peek et al., 2008). Subsequently, this biomarker was examined in the context of cardiac damage in 15 neonatal calves suffering from diarrhea induced by cryptosporidiosis, and it was observed to exhibit a slight increase (Isik and Ilhan, 2018). The study concluded that cryptosporidiosis might induce cardiac damage in calves. However, a notable limitation of the aforementioned investigation lies in the absence of an assessment about the physiopathological origin of cardiac damage in relation to sepsis. This limitation precluded the determination of whether the observed damage was a result of the causative agent itself or sepsis.

Elevations in cardiac troponins have been associated with cardiac injury resulting from conditions such as infectious myocarditis, cardiac hypertrophy, cardiac dilation, mitral valve disorders, hypotension, hemangiomas, hypovolemia, hypoxia, severe toxemia, and bacterial endotoxemia (Kroff et al., 2006). Increased concentrations of cTnI have been reported as indicators of severe myocardial injury and are associated with a poor prognosis (Mehta et al., 2004). Conversely, cTnI levels are not elevated in all patients with septic shock, which has been linked to the etiology and severity of the sepsis (Kroff et al., 2006).

Natriuretic peptides are essential for regulating fluid balance and blood pressure by suppressing the renin-angiotensin-aldosterone system. They are released in response to increased cardiac volume or vasoconstriction, resulting in cardiac relaxation, vasodilation, dilation, natriuresis, and diuresis (Chiurcihiu et al., 2008). B-type natriuretic peptides are mainly synthesized by ventricular myocytes in response to ventricular dysfunction and possess significant therapeutic, prognostic, and diagnostic value in critical conditions. Studies have shown that individuals with septic shock and severe sepsis often have elevated BNP concentrations, with higher levels correlating with a potentially worse prognosis (Lin et al., 2016). Therefore, NT-proBNP and BNP are recommended as diagnostic and prognostic biomarkers for heart failure (McMurray et al., 2012). In summary, troponins are highly specific and sensitive for identifying cardiac muscle damage, while natriuretic peptides are valuable for both diagnostic and prognostic purposes in cardiac dysfunction (Aygun and Yildiz, 2018; Ayvazoglu et al., 2023).

The concentration of cTnI in the present study differed among all the groups and was highest in the Sepsis group ($P<0.000$). Although serum NT-proBNP concentration was statistically insignificant in the comparison of Sepsis and Non-sepsis groups, it was higher in the diarrheic calves than in the healthy ones ($P<0.017$). Additionally, within the scope of the comparative ROC analysis, it was determined that the diagnostic performance of cTnI and NT-proBNP

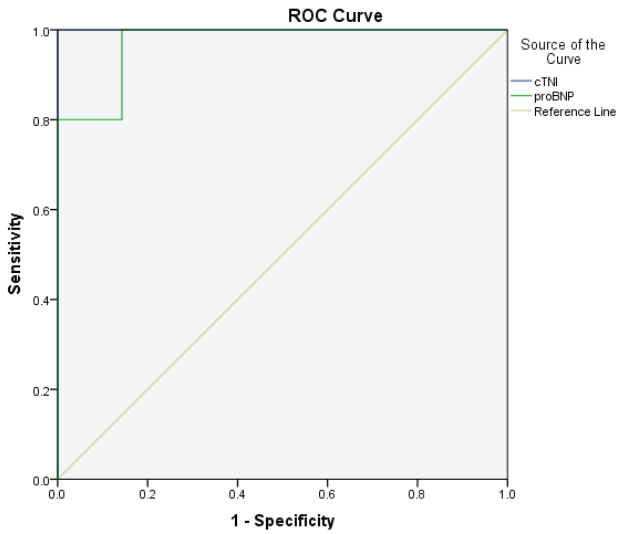

Figure 1. ROC curves of cTnI and NT-proBNP for discriminating between septic and healthy calves.

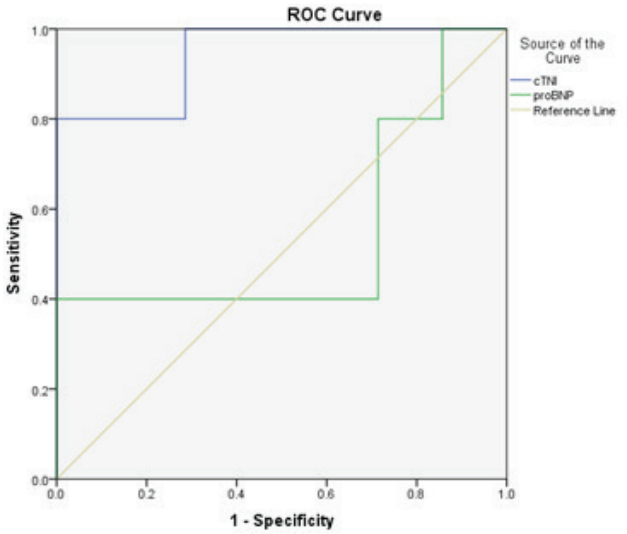

Figure 2. ROC curves of cTnI and NT-proBNP for discriminating between septic and non-septic calves.

was outstanding in distinguishing the septic calves from the healthy ones (AUC=1.000 and AUC=0.971, respectively). Similarly, it was observed that the diagnostic performance of cTnI and NT-proBNP was outstanding in distinguishing the calves without sepsis that developed diarrhea due to *Cryptosporidium* spp. from the healthy calves (AUC=0.918 and AUC=0.918, respectively) Although cTnI had an outstanding diagnostic performance in distinguishing the calves with sepsis from the non-septic ones, it was determined that NT-proBNP did not have a diagnostic discrimination performance (Table 3, 4 and 5).

Although cTnI and NT-proBNP demonstrated outstanding AUC values in distinguishing septic calves from healthy controls, their specificity in differentiating non-septic calves from healthy calves were relatively low. This suggests that while these biomarkers effectively identify cardiac dysfunction associated with sepsis, their ability to discriminate between non-septic and healthy calves at the selected thresholds is limited (Table 5). The reduced specificity may result from overlapping biomarker levels in non-septic calves, potentially reflecting subclinical conditions or physiological variability. In addition, the high variance observed in NT-proBNP levels may relate to inter-individual variability, disease stage, or renal function (Kroff et al., 2006; Drosatos et al., 2015). Consequently, further investigation and refinement of cutoff values are warranted to enhance diagnostic accuracy for differentiating non-septic from healthy calves, particularly in larger and more heterogeneous populations.

Cryptosporidium spp. cause disruption of microvilli in the intestine, villous atrophy and villous fusion. In the later stages of the disease, inflammatory changes occur (Fayer, 2010). The clinical signs are attributed to dehydration, hyponatremia, hyperkalemia, acidosis, and intestinal bacterial overgrowth due to malabsorption (Ewaschuk et al., 2002; Zello et al., 2005). In the present study, as a result of clinical examinations, higher pulse rate, CHS and degree of dehydration were determined in calves with *Cryptosporidium* spp. than in the healthy calves, regardless of the presence of sepsis. No statistical difference was detected when comparing septic and non-septic calves (Table 1). Considering that the major mechanism of *Cryptosporidium* spp. causing diarrhea is malabsorption, tachycardia is associated with intestinal bacterial overgrowth (Fadeeva et al., 2019), a high degree of dehydration is associated with rapid intestinal fluid and electrolyte loss (Naylor, 2009), and higher CHS is associated with the severity of the evaluated clinical score parameters evaluated (McGuirk, 2008). The absence of a significant difference in pulse rate, CHS, and degree of dehydration between septic and non-septic calves may be attributed to the timing of admission and the duration of symptoms in diarrheic calves. The comparable duration of symptoms in septic and non-septic calves

may account for the similar degree of dehydration and the analogous changes in pulse rate and CHS.

Cardiac damage/dysfunction in septic calves is well-described. Cardiac dysfunction is a significant consequence of sepsis that impacts mortality. It has been linked to either heightened inflammation or the suppression of fatty acid and glucose oxidation, leading to eventual ATP depletion (Drosatos et al., 2015). In our study, cardiac involvement was also observed in non-septic calves. Although the underlying mechanisms were not directly investigated, this finding might be explained by indirect effects of *Cryptosporidium* spp. on systemic inflammatory responses through intestinal barrier dysfunction, which may in turn affect cardiac tissues (Al-Sadi et al., 2009; Menard et al., 2010; Li et al., 2016; Kumar et al., 2018; Feng et al., 2019). These systemic effects might in turn influence distant organs such as the heart. However, this hypothesis remains speculative in the absence of direct assessment of intestinal damage or systemic inflammatory markers.

A culture from blood is essential for the definitive diagnosis of sepsis. One of the significant limitations of this study is the inability to perform culture from blood; thus, the mentioned sepsis criteria were utilized. We recommend that future studies confirm sepsis criteria through culture from blood.

Although small sample sizes are common in medical testing, there is a notable lack of comprehensive studies that explore various methods for constructing confidence or credible intervals (CIs) for the AUC in such scenarios. Previous studies have demonstrated that when the true AUC is high and sample sizes are small, variability in AUC estimates tends to increase (Hosmer and Lemeshow, 2000). Consequently, a key limitation of the present study is the small sample size, which may have contributed to the exceptionally high AUC values observed and could affect the reliability and generalizability of the ROC-based diagnostic performance results. This limitation is explicitly acknowledged in the discussion, along with an emphasis on the need for validation in larger cohorts.

Another limitation is that intestinal barrier dysfunction and subsequent systemic inflammatory pathway activation, which are the possible causes of cardiovascular damage in neonatal calf diarrhea due to *Cryptosporidium* spp., were not supported by the evaluation of intestinal damage markers and/or inflammation markers.

Another limitation of this study is the slight imbalance in the breed and gender distribution among the selected calves. This imbalance resulted from the strict inclusion and exclusion criteria applied to ensure monoinfection with *Cryptosporidium* spp. and significantly reduced the number of suitable cases. Due to the small sample size, the potential impact of these factors on the results cannot be completely disregarded. Future prospective studies with larger-scale and more balanced groups are warranted to confirm these findings.

CONCLUSION

These findings suggest the possibility of cardiac dysfunction even in non-septic calves with Cryptosporidium-induced diarrhea, underscoring the need for cardiac evaluation in such cases. It was concluded that cardiovascular disorders may develop in neonatal calf diarrhea due to *Cryptosporidium* spp. without sepsis. Thus, it is recommended that cardiac involvement should be considered in treatment planning and prognosis prediction in cases of neonatal calf diarrhea due to *Cryptosporidium* spp.

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CONFLICT OF INTEREST

The authors declare they have no conflicts of interest.

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