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RESEARCH ARTICLE

Differential Diagnostic Value of Serum Procalcitonin and Iron Levels in Diarrheic Neonatal Calves Caused by *Escherichia coli* and Rotavirus

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ABSTRACT

The most common enteropathogens causing diarrhea in neonatal calves are *Escherichia coli* (*E. coli*) and rotavirus. Procalcitonin (PCT) is a parameter that has recently become widely used to determine whether infectious diseases are caused by bacteria. Iron is an essential nutrient for almost all bacterial species, and serum iron levels are used as an inflammatory biomarker. Therefore, in this study, we aimed to investigate the differential diagnosis value of serum iron and procalcitonin levels in *E. coli* and rotavirus diarrhea. The material of the study consisted of 30 calves 1-15 days old. Three groups were formed as: *E. coli* (n=10), rotavirus (n=10) and control (n=10). Calves in the *E. coli* group had the highest PCT (P=0.005) and CRP (P=0.003) levels, as well as the lowest Fe (P=0.000) levels. As a result, it was determined that serum Fe levels could be used as an inflammatory marker and PCT levels higher than 50 pg/mL could be used in the differential diagnosis of *E. coli* diarrhea in calves with 100% sensitivity and 100% specificity.

Keywords: Diarrhea, *Escherichia coli*, Iron levels, Procalcitonin, Rotavirus

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Escherichia coli ve Rotavirus Kaynaklı İshalli Neonatal Buzağılarda Serum Prokalsitonin ve Demir Düzeylerinin Ayırıcı Tanı Değeri

ÖZ

Yeni doğan buzağılarda ishale neden olan en yaygın patojenler *Escherichia coli* (*E. coli*) ve rotavirüs'tür. Prokalsitonin (PCT), son zamanlarda bulaşıcı hastalıkların etiyolojisinin bakteriyel olup olmadığını belirlemek için yaygın olarak kullanılan bir parametredir. Demir (Fe) neredeyse tüm bakteri türleri için temel bir besin olup serum Fe düzeyleri yangısal bir biyobelirteç olarak kullanılmaktadır. Bu yüzden bu çalışmada *E. coli* ve rotavirüs ishallerinde serum Fe ve PCT düzeylerinin ayırıcı tanı değerini araştırmayı amaçladık. Çalışmanın materyalini 1-15 günlük 30 buzağı oluşturdu. Buzağılar *E. coli* (n=10), rotavirüs (n=10) ve kontrol (n=10) grubu olmak üzere 3 gruba ayrıldı. En yüksek PCT (P=0.005) ve CRP (P=0.003) değerleri ve en düşük Fe (P=0.000) değerleri *E. coli* grubundaki buzağılarda saptandı. Sonuç olarak, serum Fe düzeylerinin inflamatuar belirteç olarak kullanılabileceği ve 50 pg/mL'den yüksek PCT düzeylerinin buzağılarda *E. coli*'ye bağlı ishalin ayırıcı tanısında %100 duyarlılık ve %100 özgüllük ile kullanılabileceği belirlendi.

Anahtar Kelimeler: Diyare, *Escherichia coli*, Demir düzeyleri, Prokalsitonin, Rotavirüs

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INTRODUCTION

Neonatal calves are highly susceptible to bacterial, viral, and parasitic enteritis due to the immaturity of their immune systems and the failure of passive transfer of maternal antibodies (Cho and Yoon 2014). *Escherichia coli* (*E. coli*), rotavirus, coronavirus and *Cryptosporidium parvum* are the four most common enteropathogens causing neonatal calf diarrhea (Meganck et al. 2014). Nonetheless, *E. coli* and rotavirus are the most frequently isolated pathogens in diarrhea, one of the most important causes of calf mortality (Meganck et al. 2014). Pathogenic *E. coli* strains cause diarrhea, immunosuppression, intestinal wall damage, and increased bacterial load (Lofstedt et al. 2019). Furthermore, septicemia, also known as colisepticemia, frequently develops due to bacterial translocation to the bloodstream (Fecteau et al. 2009). Rotavirus is the most well-known pathogen causing acute diarrhea in calves younger than one-month-old (Alfieri et al. 2006, Barrington et al. 2002). Dehydration and rapid fluid loss occur as a result of infection's quick appearance and spread, which severely damages the intestinal lining (Cook et al. 2004).

The rapid diagnosis of pathogens is vital since determining the pathogens will affect the treatment plan and success. As is known, pro-inflammatory cytokines and acute-phase proteins begin to be produced as soon as the pathogens enter the body (Eckersall and Bell 2010). Procalcitonin (PCT), C-reactive protein (CRP), serum amyloid A, and haptoglobin are examples of positive acute phase proteins whose concentrations increase in cases of acute inflammation, while albumin and transferrin are examples of negative acute phase proteins whose concentrations decrease (Ceciliani et al. 2002, Petersen et al. 2004). In recent years, PCT, a precursor of the hormone calcitonin, has been widely used in human medicine for the early detection of bacterial diseases. The absence of an increase in PCT in human viral diseases is critical in confirming whether the infections are of viral or bacterial origin (Matur et al. 2017). Baseline PCT serum levels have been reported to increase 10-100-fold in systemic infections caused by bacterial infections (Becker et al. 2010, Carrol et al. 2002, Ruokonen et al. 2002). Furthermore, the amount of increase in PCT levels provides valuable information about the severity of the infection. (Matur et al. 2017).

Serum iron (Fe) levels have been used in both human and veterinary medicine to monitor the inflammatory process and evaluate the systemic response. Serum Fe levels decrease as infection severity increases (Ayoglu et al. 2016). This decrease has been explained as the body's defense mechanism to limit the use of serum Fe by pathogens and tumors (Weinberg and Miklossy 2008). Serum Fe concentration has been evaluated as an inflammatory biomarker in dogs, cats (Neumann 2003), horses (Borges et al. 2007), and cattle (Baydar and Dabak 2014, Değirmençay et al. 2022, Kirbas et al. 2019, Yurdakul and Aydoğdu 2020). Fe levels are reduced not only in bacterial infections (Borges et al. 2007), but also in viral infections (Değirmençay et al. 2022, Zhao et al. 2020) and non-infectious inflammations (Tsukano et al. 2019).

Serum PCT levels are highly elevated in bacterial infections, and gram-negative bacteria cause an even more significant increase in PCT levels (Zhao et al. 2020). At the same time, gram-negative bacteria require Fe to grow, and lower Fe levels may be a host defense mechanism to limit bacterial growth (Bullen 1981). As a result, we hypothesized that serum PCT levels in calves with *E. coli* would be quite high and Fe levels relatively low when compared to those with rotavirus in this study. Second, we hypothesized that serum PCT and Fe levels could be useful indicators for the differential diagnosis of *E. coli* and rotavirus diarrhea.

MATERIALS and METHODS

This study was carried out in accordance with Atatürk University's approved ethical rules (protocol no. 2022/2, decision number: 38, date: 02/28/2022), and written informed consent was obtained from the owner for each calf.

Animals and Protocol Design

The study material included 30 cattle, 1-15 days old, Simmental and Montofon breeds, and both genders. The diarrheic calves were divided into two groups based on rapid test kit (Rapid BoviD-5 Ag Test Kit; Cat. No: RC1302DD) results: *E. coli* (n=10) and rotavirus (n=10). Calves with normal clinical examination and haematological findings were assigned to a control group (n=10). All calves' rectal temperatures (RT), heart rates (HR), and respiratory rates (RR) were measured and recorded during the clinical examination.

Blood Sampling

Blood samples from all the calves were taken from vena jugularis externa and collected into tubes with EDTA (Vacutainer, K2E 3.6 mg, BD, UK) and gel (Vacutainer, BD, UK) for haematological and biochemical analyses. Blood samples in gel tubes were kept at room temperature before being centrifuged at 3000 rpm for 10 minutes. The obtained serum samples were stored at -80°C until biochemical analysis.The haematological analyses were completed immediately.

Haematological Analyses

White blood cell (WBC), lymphocyte (LYM), monocyte (MON), neutrophil (NEU), eosinophil (EOS), basophil (BAS), red blood cell (RBC), and haemoglobin (HGB) counts, haematocrit (HCT) and platelet (PLT) levels of the cattle were determined by

a haematology analyser (Abacus Junior Vet5, Hungary).

Biochemical Analyses

Serum PCT concentrations were determined by the electrochemiluminescence immunoassay (ECLIA) method using a chemistry analyser (Cobas e801; Roche Diagnostics, Switzerland). CRP serum concentrations were measured using the latex-enhanced immunoturbidimetric assay (Cobas c702; Roche Diagnostics, Switzerland). Serum Fe, blood urea nitrogen (BUN), creatinine (CREA), total protein (TP), albumin (ALB), and globulin (GLOB) concentrations in serum samples were determined by commercial kits using a biochemistry autoanalyzer (Mindray BS-300 Chemistry Analyser, China).

Statistical Analyses

The SPSS software program (Version 25.0, SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The Shapiro-Wilk test was used to determine the variables' normality and the variances' homogeneity. Nonparametric data were evaluated using the Kruskal Wallis H test as median (minimum/maximum). The Spearman correlation test was used to detect the correlation between variables. Receiver operating characteristic (ROC) curve analyses were made using Medcalc version 20 software (Mariakerke, Belgium). The diagnostic values of Fe and PCT were evaluated using ROC curve analysis to determine the diagnostic cut-offs for the best differentiation between bacterial and viral infections. All statistical comparisons were performed at the significance level of $P<0.05$

RESULTS

Clinical Findings

The *E. coli* and rotavirus groups had a low temperature and increased respiratory frequency. Calves generally showed varying degrees of diarrhea, dehydration, weakness, appetite loss, and diminished sucking reflex.

Hematological Findings

In the analysis of haematological parameters, calves in the rotavirus group had higher WBC (P=0.006), NEU (P=0.004), and PLT (P=0.000) values than calves in control and *E. coli* groups. The RBC (P=0.002), HGB (P=0.001), and HCT (P=0.000) values of the calves in the rotavirus and *E. coli* groups were higher than those in the control group, consistent with dehydration (Table 1).

Biochemical Findings

When the biochemical parameters of the groups were examined, the Fe levels were found to be low in both patient groups (P=0.000). The highest PCT (P=0.000) and CRP (P=0.003) values and the lowest Fe (P=0.000) values were determined in calves in the *E. coli* group. The BUN (P=0.000), CREA (P=0.001), TP (P=0.002), ALB (P=0.002), and GLOB (P=0.035) values were significantly higher in the patient groups than in the control group (Table 1).

All groups' correlation results showed that serum PCT levels were strongly negatively correlated with Fe levels (r=-0.663, p=0.000). Serum Fe levels were moderately negatively correlated with NEU (r=-0.422, p=0.020), while it was weakly negatively correlated with CRP (r=-0.384, p=0.036) (Table 2). HCT levels were strongly positively correlated with BUN (r=0.716, p=0.000) and CREA levels (r=0.644, p=0.000).

Serum PCT and Fe were shown to have a strong negative correlation (r=-0.802, p=0.000) based on the correlation results between the *E. coli* and control groups. The correlation between PCT and CRP (r=0.569, p=0.009) and NEU (r=0.577, p=0.008) was moderately positive. Fe and CRP showed a strong negative correlation (r=-0.612, p=0.004) (Table 3).

The correlation results between the rotavirus and control groups revealed a highly negative correlation between serum Fe and WBC (r=-0.771, p=0.000) and NEU (r=-0.746, p=0.000). A moderate negative correlation between PCT and Fe was found in the correlation analysis between *E. coli* and rotavirus groups (r=-0.516, p=0.020).

ROC analysis results of Fe, PCT, and CRP between control and *E. coli* groups are shown in Table 4 and Figure 1. The areas under the ROC curves (AUC) were 1.000 for the Fe and PCT and 0.925 for the CRP parameter. The cut-off values of Fe and PCT parameters for showing bacterial infection were ≤56.59 μg/dL, and >50 pg/mL, respectively. The sensitivity and specificity values of the proposed diagnostic cut-off point for demonstrating bacterial infection were 100% and 100% for Fe and PCT.

ROC analysis results of Fe, PCT, and CRP between control and rotavirus groups are shown in Table 5 and Figure 2. The areas under the ROC curves (AUC) were 1.000 for the Fe, 0.560 for the PCT, and 0.820 for the CRP parameter. The cut-off values of Fe and PCT parameters for showing viral infection were ≤89 μg/dL, and >40 pg/mL, respectively. The proposed diagnostic cut-off points for demonstrating viral infection had sensitivity and specificity values of 100% and 100% for Fe and 30% and 90% for PCT, respectively.

Table 1. Comparison of haematological, biochemical and some clinical parameters of calves in control, *E. coli* and

Parameters	Control group (n:10)	E. coli group (n:10)	Rotavirus group (n:10)	P value
WBC (x10³/µL)	8.645^{A} (7.65-9.83)	10.21^{AB} (5.23-29.8)	**17.05^{B} (11.28-26.68)**	**0.006**
LYM (x10³/µL)	4.68^{A} (3.08-5.83)	3.605^{A} (2.37-7.38)	4.86^{A} (2.75-10.56)	0.117
MON (x10³/µL)	0.215^{A} (0.12-0.42)	0.11^{B} (0.04-0.54)	0.195^{A} (0.1-1.48)	0.036
NEU (x10³/µL)	3.665^{A} (2.57-5.3)	7.465^{AB} (0.06-23.59)	**11.13^{B} (7.77-20.71)**	**0.004**
EOS (x10³/µL)	0.035^{A} (0.01-0.05)	0.02^{A} (0-0.1)	0.03^{A} (0.01-0.12)	0.543
BAS (x10³/µL)	0^{A} (0-0)	0.01^{B} (0-0.05)	0.03^{C} (0.01-0.16)	0.000
RBC (x10⁶/µL)	8.225^{A} (7.33-8.9)	**9.475^{B} (8.11-13.56)**	**10.445^{B} (7.94-13.99)**	**0.002**
HGB (g/dL)	9.5^{A} (9.1-9.7)	**11.45^{B} (9.8-15.9)**	**13.85^{B} (9-16.9)**	**0.001**
HCT (%)	26.36^{A} (24.48-27.88)	**38.775^{B} (33-56.75)**	**45.165^{B} (30.38-53.14)**	**0.000**
PLT (x10³/µL)	369.5^{A} (277-443)	319.5^{A} (261-520)	**627.5^{B} (453-769)**	0.000
Fe (µg/dL)	151.7^{A} (96.6-297.1)	**21.44^{B} (10.95-56.59)**	**41.99^{B} (7.71-89)**	**0.000**
PCT (pg/mL)	20^{A} (20-50)	**115^{B} (80-240)**	20^{A} (20-54)	**0.000**
CRP (Mg/L)	0.06^{A} (0-0.07)	**0.11^{B} (0.07-0.18)**	0.08^{B} (0.04-0.24)	**0.003**
BUN (mg/dL)	16^{A} (10-23)	**62.36^{B} (4.87-172.36)**	**126.305^{B} (46.23-151.38)**	**0.000**
Creatinine (mg/dL)	0.76^{A} (0.55-4.62)	**3.96^{B} (1.11-6.45)**	**2.68^{B} (1.03-7.03)**	**0.001**
TP (g/dL)	4.85^{A} (4.1-5.6)	**6.95^{B} (4.98-11.27)**	**7.11^{B} (1.72-8.71)**	**0.002**
ALB (g/dL)	2.05^{A} (1.74-2.19)	**2.65^{B} (2-3.8)**	**2.6^{B} (0.8-3.34)**	**0.002**
GLOB (g/dL)	2.82 ^{A} (1.9-3.9)	**4.35^{B} (1.9-8.6)**	**4.07^{B} (0.9-6.4)**	**0.035**
RT (°C)	38.85 (38.4-39.3)	38.55 (38.1-39.1)	38.15 (36-39.8)	0.324
HR (beats/min)	132 (92-176)	136 (76-160)	118 (58-156)	0.335
RR (breaths/min)	28 (20-72)	36 (28-72)	38 (18-56)	0.112

rotavirus groups

WBC: white blood cell; LYM: lymphocyte; MON: monocyte; NEU: neutrophil; EOS: eosinophil; BAS: basophil; RBC: red blood cell; HGB: haemoglobin; HCT: haematocrit; PLT: platelet; Fe: iron; PCT: procalcitonin; CRP: C-reactive protein; BUN: Blood urea nitrogen; CREA: Creatinine; TP: Total protein; ALB: Albumin; GLOB: Globulin; RT: Rectal temperature; HR: Heart rate (per min); RR: Respiratory rate (per min). Data are presented as median (range). Different letters in the same line are statistically significant (P<0.05).

Table 2. Correlation results between PCT and Fe, CRP, WBC, MON, and NEU levels in diarrheic and healthy calves (Spearman correlation analysis)

Parameters	PCT	Fe	CRP	WBC	MON	NEU
PCT	1.000	-0.663**	0.214	0.103	-0.264	0.253
Fe		1.000	-0.384*	-0.351	-0.135	-0.422*
CRP			1.000	0.052	-0.264	0.101
WBC				1.000	0.219	0.926**
MON					1.000	0.061
NEU						1.000

PCT: procalcitonin; Fe: iron; CRP: C-reactive protein; WBC: white blood cell; MON: monocyte; NEU: neutrophil. *P <0.05, **P<0.01.

Table 3. Correlation results between PCT and Fe, CRP, WBC, MON, and NEU levels in *E. coli* and control groups (Spearman correlation analysis)

Parameters	PCT	Fe	CRP	WBC	MON	NEU
PCT	1.000	-0.802**	0.569**	0.319	-0.377	0.577**
Fe		1.000	-0.612**	-0.042	0.357	-0.233
CRP			1.000	-0.113	-0.461*	0.025
WBC				1.000	0.143	0.798**
MON					1.000	-0.045
NEU						1.000

PCT: procalcitonin; Fe: iron; CRP: C-reactive protein; WBC: white blood cell; MON: monocyte; NEU: neutrophil. *P <0.05, **P<0.01.

Table 4. Receiver operating characteristic (ROC) results of Fe, PCT and CRP between control and *E. coli* groups (n=20)

Parameters	Fe (μg/dL)	PCT (pg/mL)	CRP (Mg/L)
Area	1.000	1.000	0.925
Cut-off	≤56.59	>50	>0.07
Sensitivity (%)	100	100	70
Specificity (%)	100	100	100
SEM	0.000	0.000	0.0456
P value	<0.001	<0.001	<0.001

Fe: iron; PCT: procalcitonin; CRP: C-reactive protein

Table 5. Receiver operating characteristic (ROC) results of Fe, PCT and CRP between control and rotavirus groups (n=20)

Parameters	Fe (μg/dL)	PCT (pg/ml)	CRP (Mg/L)
Area	1.000	0.560	0.820
Cut-off	≤89	>40	>0.07
Sensitivity (%)	100	30	50
Specificity (%)	100	90	100
SEM	0.000	0.103	0.0896
P value	<0.001	0.559	<0.001

Fe: iron; PCT: procalcitonin; CRP: C-reactive protein

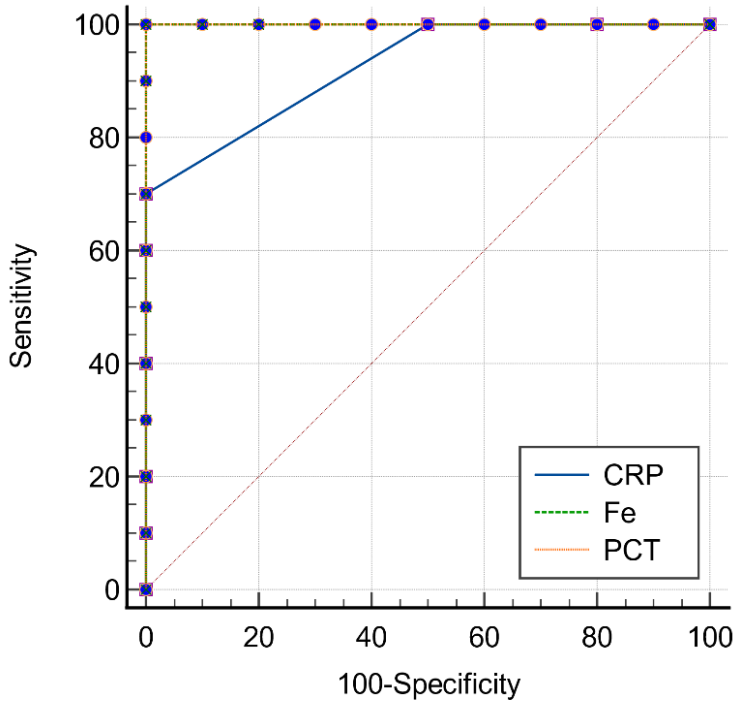

Figure 1. ROC curve analysis of Fe, PCT and CRP between control and *E. coli* groups

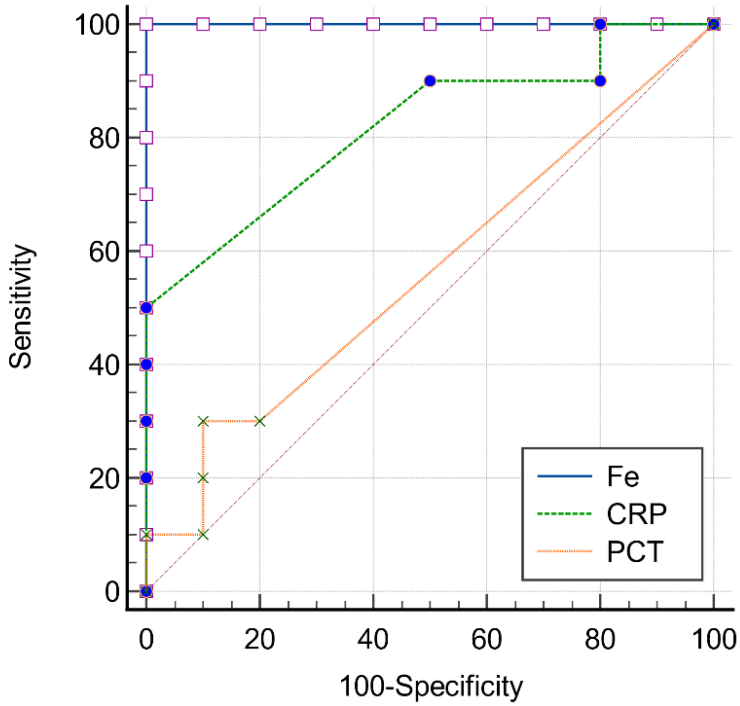

Figure 2. ROC curve analysis of Fe, PCT and CRP between control and rotavirus groups

DISCUSSION

In this study, we investigated the utility of serum PCT and Fe levels in the differential diagnosis of calves with diarrhea due to *E. coli* and rotavirus. Consistent with our hypothesis, the *E. coli* group had the highest PCT and lowest Fe levels in calves. We also suggested that PCT levels >50 pg/mL and Fe levels less than ≤56.59 μg/dL could be used with 100% sensitivity and 100% specificity in the differential diagnosis of *E. coli* diarrhea in calves.

It has been reported that PCT concentrations in neonatal calves with septicemic colibacillosis are approximately four times higher than in the control group. There is a positive correlation between PCT and proinflammatory cytokines. As a result, PCT has been suggested as a useful marker of septicemic colibacillosis in neonatal calves (Ercan et al. 2016). Similarly, very high PCT levels were found in dogs given endotoxin (Easley et al. 2020), horses with SIRS (Bonelli et al. 2015), and horses with colic with endotoxemia (Teschner et al. 2015). In our study, PCT levels in the *E. coli* group were five times higher than in the control group. In ROC analysis, the diagnostic cut-off value of PCT levels was found to be >50 pg/mL with 100% sensitivity and specificity for bacterial infection. Therefore, our results suggest that PCT could be used as a potential diagnostic marker of *E. coli* diarrhea in calves.

Procalcitonin has been shown to be elevated in bacterial infections but remained low in viral infections (Gendrel et al. 1999, Simon et al. 2004). Similar to this, we noticed that PCT levels in the rotavirus group were the same as in the control group and did not rise. Bacterial endotoxins are the primary trigger for PCT induction (Easley et al. 2020, Lippi and Cervellin 2018). As is well known, Enterotoxigenic *E. coli* (K99 - ETEC) is the predominant strain that causes neonatal calf diarrhea. They produce infectious diarrhea by directly attaching to enterocytes in the intestinal mucosa or by inducing inflammation with their toxins or lipopolysaccharides (Foster and Smith 2009). These arguments suggest that our hypothesis regarding elevated PCT levels in the *E. coli* group is rather logical. PCT levels can be used as a potentially effective marker to distinguish *E. coli* diarrhea from rotavirus diarrhea, given that the sensitivity and specificity of PCT in ROC analysis are both 100%.

In severe systemic inflammation or bacterial infections, PCT is noticeably enhanced (up to 5.000-fold) within 2 to 4 hours, and the level lasts until recovery (Gilbert 2010, Pfäfflin and Schleicher 2009). CRP and WBC, two inflammatory biomarkers, are not specific for bacterial infections (Müller et al. 2007). PCT is superior to CRP and other acute phase reactants because of its biological half-life of 22 to 26 hours (Limper et al. 2010). This study found a moderately strong positive correlation between PCT and CRP, and NEU in the correlation results between the control and E. coli groups. Although there was a non-significant increase in CRP and NEU levels in both patient groups, there was a statistically significant PCT elevation in the *E. coli* group. This suggests that PCT levels are a good marker that can be used to distinguish *E. coli* diarrhea from rotavirus diarrhea. Serum PCT levels are reported to decrease after administering the appropriate antibiotic treatments (Assicot et al. 1993). Serum PCT levels can help clinicians decide whether to start or stop antibiotic therapy (Covington et al. 2018, Wolfisberg et al. 2022). We argue that PCT can be used effectively in diagnosing bacterial infections in calf diarrhea and as a marker to guide antibiotic therapy.

Serum Fe levels tend to decline as infection severity rises and hence can be used as a marker for inflammation (Baydar and Dabak 2014, Değirmençay et al. 2022, Tsukano et al. 2020). Similarly, in this study, serum Fe levels decreased and negatively correlated with PCT, CRP, and NEU levels. Therefore, serum Fe levels can be used as a marker of inflammation in calves with neonatal diarrhea. The *E. coli* group had the lowest serum Fe levels, yet the rotavirus group also declined. This can be explained by the decrease in serum Fe levels not only in bacterial infections but also in viral and non-infectious inflammation conditions. Decreased Fe levels will diminish the utility of serum Fe levels in the differential diagnosis of viral infections. Nevertheless, based on the findings of our ROC analysis, we can state that Fe levels between 56.59 and 89 μg/dL will be used to diagnose rotavirus diarrhea with 100% sensitivity and 100% specificity. In contrast, Fe levels below 56.59 μg/dL are used to diagnose *E. coli* diarrhea with 100% sensitivity and 100% specificity.

CONCLUSIONS

In conclusion, we investigated the changes in serum PCT and Fe levels in calves with *E. coli* and rotavirus diarrhea, as well as the utility of these markers in differential diagnosis. While serum Fe levels decreased in both patient groups, we found that serum PCT levels increased excessively only in the *E. coli* group. As a result, while serum Fe levels can be used as an inflammation marker, only serum PCT levels can be used in differential diagnosis. Based on the intergroup comparison and ROC analysis, PCT levels greater than 50 pg/mL have 100% sensitivity and 100% specificity in the differential diagnosis of *E. coli* diarrhea in calves.

Ethics Committee Information: This study was carried out in accordance with Atatürk University's approved ethical rules (protocol no. 2022/2, decision number: 38, date: 02/28/2022), and written informed consent was obtained from the owner for each calf. The information, data, and documents presented in

this article were obtained in accordance with academic and ethical rules.

Conflict of Interest: The authors declare no conflict of interest.

Authors Contribution Rate: ŞD: %70, MSE: %15, EE: %15

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