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Procalcitonin As a Metric Septic Inflammatory Biomarker in Young Calves

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ABSTRACT

Septicemia in large animals is one of the common causes of mortality with the difficulties of early diagnosis. To diagnose sepsis in animals, clinical signs and blood cultures are combined with hematological values, inflammatory biomarkers and serum biochemistry. Biomarkers specific to sepsis, such as procalcitonin (PCT), have been the subject of recent research in humans and animals. This study evaluated PCT concentration using enzyme-linked immunosorbent assay (ELISA) in serum samples collected from 50 calves with clinical septicemic disease and 20 healthy calves. PCT concentration was 66.86±0.15 pg/mL (25.4-140.2 pg/mL) in the control healthy group and 756.1±3.65 pg/mL (90.23-2185 pg/mL) in the diseased group. The optimal cutoff value for distinction between healthy and sick calves was 148.4 pg/mL. Using a receiver operator characteristic (ROC curve) revealed the highest area under the curve (AUC) value of procalcitonin 0.99; 95% confidence interval [CI]. ELISA procalcitonin test shows high diagnostic sensitivity, specificity, and accuracy for diagnosis of septicemia, and the result emphasized the importance of ELISA procalcitonin as a faster, more accurate, and metrically efficient diagnosis for sepsis in calves.

Key words: Calves, ELISA, Procalcitonin and Septicemia.

INTRODUCTION

Sepsis is a global health problem and remains one of the main causes of mortality in critically ill patients (Mellhammar *et al*., 2016). Definition of sepsis is failure of body organs due to uncontrolled infection (Seymour *et al*., 2016; Singer *et al*., 2016). Septicemia is either epidemic or sporadic in calves, which can reach 30% if predisposing factors are present. (Fecteau *et al*., 2009). Pathophysiologic changes associated with inflammation in septicemia as dehydration, generalized weakness, body temperature, respiratory and heart rate changes, as well as leukopenia and hypotension (House *et al*., 2015). Using blood culture as a diagnostic method for sepsis, the sensitivity can be low and a negative result should be interpreted with caution (Fecteau *et al*., 2009). Rapid diagnosis and antibiotic treatment are significant for the Recovery of the septic patient (Opal and van der Poll, 2015). Procalcitonin serum level is raised after bacterial infection by 2-4 hr. and reaches the highest level within 6-24 hr. and remains for 7 days so it may be useful as an aseptic marker and monitor for antibiotic therapy effectiveness and duration (Rojas-

Moreno and Regunath, 2016).

Procalcitonin is a prohormone of calcitonin protein that consists of 116 amino acids with 13 kDa (Lee, 2013). As a septic biomarker, Procalcitonin can monitor treatment duration and the prognosis of the case, as an increased level of procalcitonin indicates a poor prognosis but a decreased level of PCT indicates good prognosis and effective treatment (Mehanic and Baljic, 2013). Procalcitonin is one of the immune system components and is affected by cytokines, in infection PCT is released from parenchymal cells (Liu *et al*., 2015). Procalcitonin concentration is affected by the severity and prevalence of bacterial infections. And in viral infections, the increase of interferon-gamma suppresses serum PCT levels which can differentiate bacterial from viral infections (Schuetz *et al*., 2011 and Briel *et al*., 2008).

Bacterial load increases procalcitonin concentration in serum and also the type of bacteria; in gram- negative bacterial infections, PCT levels increased more than gram- positive bacterial infections (Leli *et al*., 2015). In veterinary medicine, C-reactive protein, haptoglobin, lactate and pro-inflammatory cytokines are used to diagnose septicemia, but their half-life are short, with low specificity, low specificity, and limited plasma stability, whereas PCT has a long half-life (20–30 hr.), is stable in specimens, has high sensitivity, and has a high response to septicemia (Carrol *et al*., 2002 and Lee, 2013).

Procalcitonin is used in multiple clinical areas of humans, but its use in veterinary medicine is limited. There are a few studies of researches on PCT in animals, although the first discovery of calcitonin was in dogs (Copp and Cheney, 1962).Afterward in experimental models such as pigs, rats and baboons. There are a few studies on ruminants and there is no difference in PCT levels between genders in healthy cattle (Ercan *et al*., 2016). Procalcitonin is valuable biomarker for sepsis in dogs and it may play a role in prognostication and therapeutic decision making (Frankie *et al*., 2019). The aim of this work was to study the role of procalcitonin as a diagnostic and prognostic biomarker in septicemic calves.

MATERIAL AND METHODS

Animals:

Fifty calves more than 4 weeks of age with septicemia and 20age-matched clinically healthy control calves from dairy farms at the period of winter and spring season in Dakahlia governorate, Egypt, were included in this study. Diagnosis of septicemia in calves was done according to clinical findings (House *et al*., 2015), isolation of pathological agents from blood, hematological and biochemical parameters alterations. Based on clinical examination, septicemic calves had variable body temperature, dehydration, weakness, hyperpnoea, anorexia, conjunctival hyperemia, and tachycardia. And healthy control calves showed no any abnormal clinical findings without hematological and biochemical alterations. The study was approved by number: 117/2017, from The Local Ethics Committee for Animal Experiments at the Faculty of Veterinary Medicine, University of Sadat City.

Sampling:

Blood samples were collected from each calf from jugular vein, blood sample was collected from each calf by 20 ml syringe into EDETA, Plain tubes (BD, UK), and blood-culture bottles (Bactec plus Aerobic/F-bioMérieux).For Hematological analyses, Biochemistry , proinflammatory and PCT values determination and Bacterial isolation .

Before venipuncture, the hair was shaved, and the site was cleaned with a povidone-iodine (10%) detergent followed by isopropyl alcohol (70%) swabbing. blood samples in Plain tubes from each calf were

left to clot and centrifuged (10 min at 4,000 rpm). Serum was aliquoted and stored at −20°C until used.

Bacterial isolation and identification:

Immediately after sample collection, blood culture bottles (Bactec plus Aerobic/F-bioMérieux) were inoculated with 10 mL of blood using a new needle after disinfection the top of the bottle with an alcohol wipe as the manufacturer's recommendation, and stored at ambient temperature until arrival to the laboratory. Bottles incubated in BACT/ALERT 3D (bioMérieux, USA) Automated microbial detection system and examined periodically for evidence of bacterial growth during the incubation period (7 days). The positive bottle was identified and removed when evident turbidity was observed or if alarm was obtained (Kireçci *et al.,* 2010). Blood broth was then aspirated aseptically for Gram staining. Blood broth mixture bottles were subcultured on MacConkey agar (Merck- Germany) and 5 % sheep blood agar (Merck- Germany) and then incubated aerobically. Microorganisms isolated from positive cultures were identified by conventional biochemical methods (Quinn *et al.,* 1994). After 7 days negative blood-culture bottles were plated on the same media as the positive samples to ensure negative bacterial growth. Isolation of a pure culture of bacteria confirms a clinical diagnosis of bacterial sepsis.

Hematological analyses:

Erythrocytes, leukocytes count and packed cell volume were evaluated by automatic cell counter (Abacus -Junior Vet5, Hungary).

Biochemistry and pro-inflammatory values determination:

Serum Creatinine, total protein, total bilirubin, albumin, and globulin concentrations were evaluated by Mindray Vet-30 chemistry analyzer (Mindray - China).

Serum IL-8, PGE2 (Sunred Biological Technology, China), and TNF-α (Cusabio Biotic, china) ELISA kits were evaluated using an automated enzyme-linked immunosorbent assay reader, Stat Fax® 4200 (Awareness Technology, Inc. USA.)

Serum PCT concentration evaluation:

Procalcitonin concentration levels in sera were evaluated by (a commercial bovine procalcitonin ELISA Kit, cat No MBS706592 My BioSource), based on a quantitative sandwich enzyme immunoassay technique. Test analytical sensitivity in cattle was evaluated as less than 9.77 pg/ml. by the manufacture, detection range of 39.06 pg/ml-2500 pg/ml. a standard curve of procalcitonin ELISA kits was established by my-Assays program Table 1, and Figure 1.

Statistical analyses:

Statistical analyses by IBM, and SPSS Statistics (29) program were performed to compare between the two groups by t-tests.

RESULTS

The clinical examination findings in healthy controlled calves were within the normal reference range, while diseased calves showing signs of septicemia which include, high pulse rate, increased respiratory rate, respiratory disorders, diarrhea and dehydration. Compared to healthy controlled. Respiratory and heart rates were significantly ($P<0.05$) higher and the body temperature was comparable ($P>0.05$) between the 2 groups (table 2 and figure2).

Microbiological blood assay:

Fifty blood cultures were performed for all diseased positive PCT for detection and isolation of causative bacteria, 24 Cases (48 % of all blood cultures) were culture positive and 26 cases (52%) were culture negative, *Table 3*. *E. coli* was isolated from 14 calves with positive blood cultures, other bacteria isolated were *Klebsiella* spp. (n=3), *B. Hemolytic streptococcus*. (n=4),

staphylococcus aureus (n=2), and *Pasteurella haemolytica*. (*n*=1), *Table 4, and Figure 3.*

Hematological values:

Septicemic calves had higher packed cell volume and leukocyte count than healthy calves (p value < 0.005). Table 5 and Figure 4.

Serum biochemistry values:

Statistical analysis of serum biochemistry values in septicemic calves found very high significant (P<0.005) increase in the values of serum total bilirubin (4.16 ± 0.32 mg/dl) compared to healthy controlled calves (0.18 ±0.02 mg/dl) and significant (P<0.001) decrease in the values of total serum protein in septicemic calves (6.6 ±0.11 gm/dl) compared with healthy controlled calves (6.97 ±0.13 gm/dl) (table 6 and figure 5).

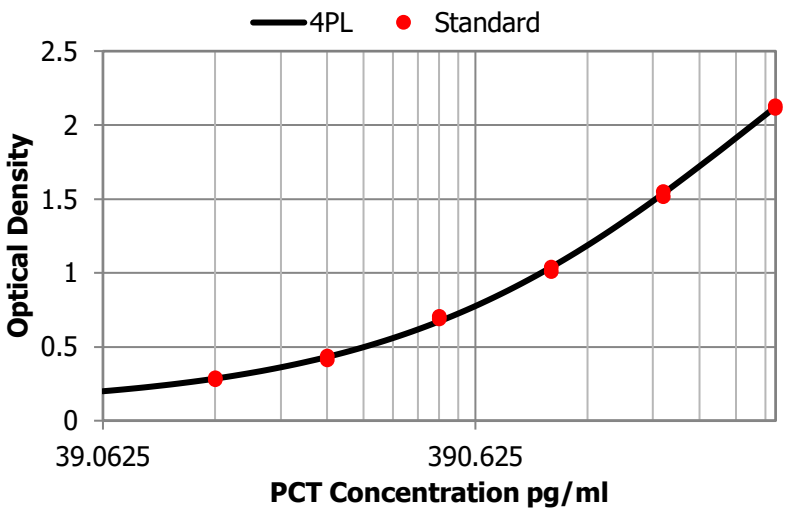
Serum procalcitonin and pro-inflammatory cytokine concentration:

Serum PCT concentration in healthy and diseased calves were 66.86 ± 0.15 pg /ml (25.4 – 140.2 pg/ml) and 756.1 ±3.65 pg/ml (90.23 – 2185 pg/ml), respectively. To differentiate healthy calves from sick calves, the optimal cut-off value was 148.4 pg/mL., Table 5. Using a receiver operator characteristic (ROC curve) revealed the highest area under the curve (AUC) value of procalcitonin 0.99; 95% confidence interval [CI]. Table 8.

In the diseased calves group, the serum procalcitonin concentration was significantly higher than healthy control group (p < 0.005), with a positive correlation between pro-inflammatory cytokines and PCT. In calves with septicemia, there were measurable increases in PGE2, IL8, and TNF concentrations. Higher levels of PGE2 were linked to elevated levels of IL-8, Table 9 and Figure 6.

Table 1: Standard optical density of PC ELISA kits.

Calibrator	Wells	Conc.	Raw	SEM	Backfit	Recovery %	Corrected OD
Standard1	A1	2500	2.13	0.0085	2532	101.3	2.117
	A2		2.12		2482	99.29	
Standard2	B1	1250	1.55	0.016	1270	101.6	1.519
	B2		1.52		1220	97.6	
Standard3	C1	625	1.04	0.015	627.8	100.5	1.012
	C2		1.01		598.3	95.73	
Standard4	D1	312.5	0.708	0.0095	338.8	108.4	0.689
	D2		0.689		324.7	103.9	
Standard5	E1	156.3	0.439	0.012	160.7	102.8	0.415
	E2		0.415		146.9	94.04	
Standard6	F1	78.13	0.291	0.0065	81.29	104	0.278
	F2		0.278		74.96	95.95	
Standard7	G1	39.06	0.185	0.0065	33.1	84.74	0.172
	G2		0.172		27.79	71.15	
Standard8	H1	0	0.105	0.002	3.618	-	-
	H2		0.101		2.45	-	



a	0.09109
b	0.8719
c	2352
d	4.05
MS	0.00028
E	97
R^2	0.9994
SS	0.00463
	6
SY	0.01965
X	

Figure 1: Standard curve of PCT ELISA kits by My-Assays program.

Table 2. The clinical findings of healthy and septicemic calves

Measurements	Healthy Calves (n=20)	Septicemic calves (n=50)
Body temperature °C	**38.78±0.05**	**39.46±0.15**
Respiratory rate (Cycle/min)	**43.10±2.15**	**58.11±0.21*****
Heart rate (beat/min)	**85.03±1.92**	**98.35±0.42*****

*** p< 0.005

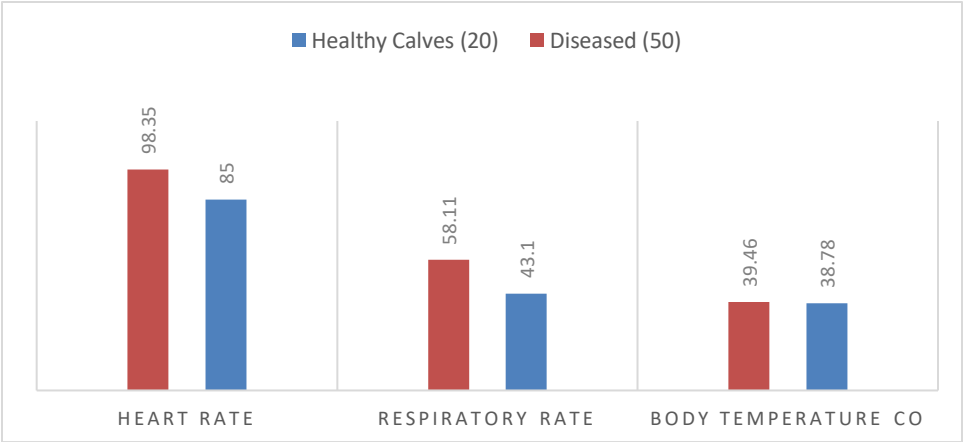


Figure 2: The clinical examination findings in calves.

Table 3: Microbiological blood assay of diseased calves positive Procalcitonin (n=50)

Parameters	+ve Culture	- ve Culture
Procalcitonin +ve (n=50)	**24 (48%)**	**26 (52%)**

Table 4: Bacterial species isolated from positive blood cultures (n=24).

Bacterial species	Positive blood cultures (n=24)	
	No	%
E. coli	**14**	**58.33**
Klebsiella spp.	**3**	**12.5**
ß.Hemolytic streptococcus	**4**	**16.67**
Staphylococcus aureus	**2**	**8.33**
Pasteurella hemolytica	**1**	**4.16**

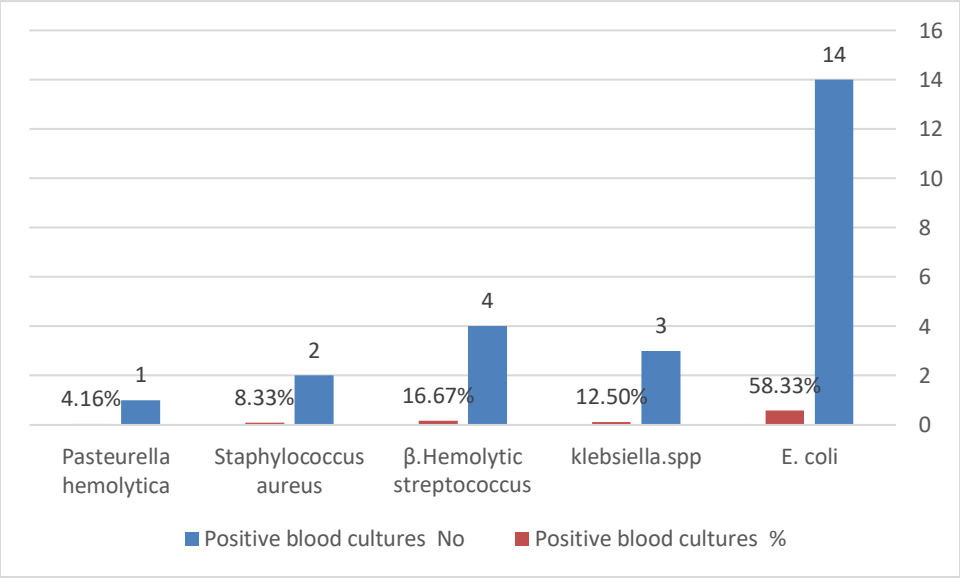


Figure 3: Bacterial species isolated from positive blood cultures (n=24).

Table 5. Hematological values in healthy control and septicemic calves.

Measurements	Healthy Calves (n=20)	Septicemic calves (n=50)
Packed cell volume %	**32.26±0.23**	**47.42±0.38*** **
Erythrocyte (10^6/µl)	**6.03±0.27**	**7.16±0.37***
Leukocyte (10^3/µl)	**5.37±1.33**	**13.42±3.16****

**** p < 0.05*** ***** p < 0.01*** ****** p < 0.005***

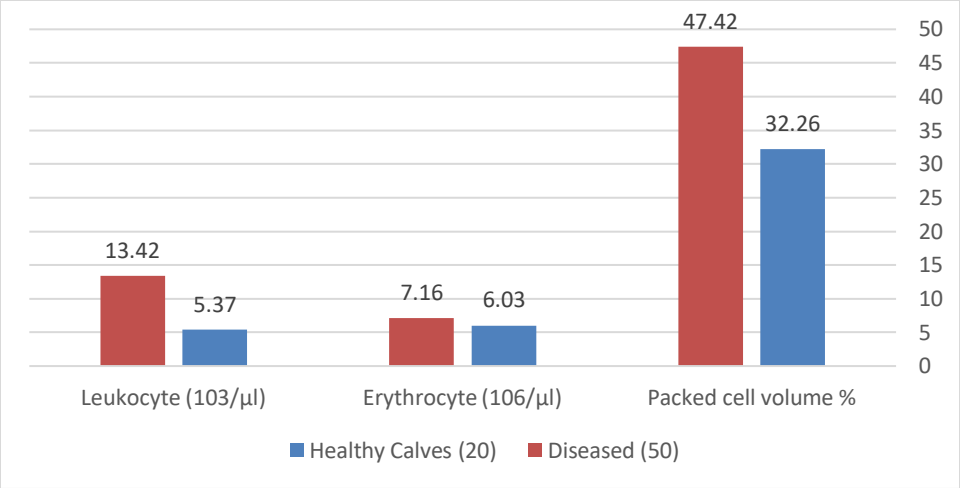


Figure 4. Hematological values in healthy control and deceased calves.

Table 6. Serum biochemistry evaluations in healthy and septicemic calves.

Parameters	HealthyCalves (n=20)	Septicemic (n=50)
Creatinine (mg/dL)	**1.07±0.84**	**2.15 ± 1.18**
Total Bilirubin (mg/dL)	**0.18±0.02**	**4.16 ± 0.32*****
Total Protein (g/dL)	**6.97±0.13**	**6.60 ± 0.11****
Albumin (g/dL)	**3.28±0.22**	**3.12 ± 0.46**
Globulin (g/dL)	**3.69±0.43**	**3.38 ± 0.57**

** $p < 0.01$ *** $p < 0.005$

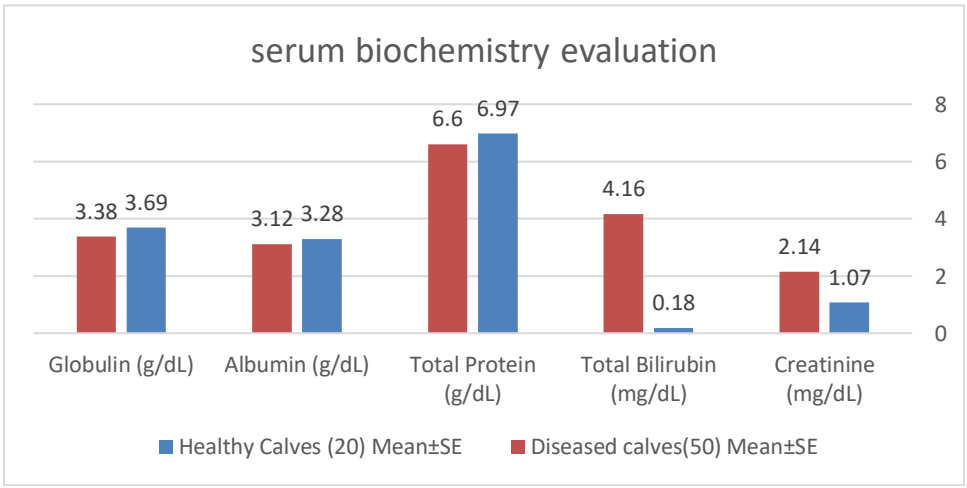


Figure 5. Serum biochemistry evaluations determined in the study.

Table 7: Procalcitonin ELISA kit Cutoff value.

Classifier Evaluation Metrics		
Test Result Variable(s): procalcitonin concentration		
Gini Index	K-S Statistics	
	Max K-S[a]	Cutoff[b]
,994	,980	148,40
a. The maximum Kolmogorov-Smirnov (K-S) metric. Also the maximum value of Youden's index.		
b. In case of multiple cutoff values associated with Max K-S, the largest one is reported.		

Table 8: Area under the ROC Curve for procalcitonin

Area Under the ROC Curve				
Test Result Variable(s): procalcitonin concentration				
Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
,997	,004	,000	,990	1,004
a. Under the nonparametric assumption				
b. Null hypothesis: true area = 0.5				

Table 9. Procalcitonin and pro-inflammatory cytokines Correlation in healthy and septicemic calves.

Parameters	**Healthy Calves (20)**	**Septicemic Calves (50)**
Procalcitonin (pg/ml)	**66.86±1.15**	**756.1±3.65*** **
PGE2 (pg/ml)	**123.43±3.61**	**127.41±2.13***
IL-8 (pg/ml)	**191.42±2.83**	**196.17±4.02**
TNF-α (ng/ml)	**0.69±0.13**	**1.08±0.32***

** p< 0.05 ** p < 0.01 *** p < 0.005*

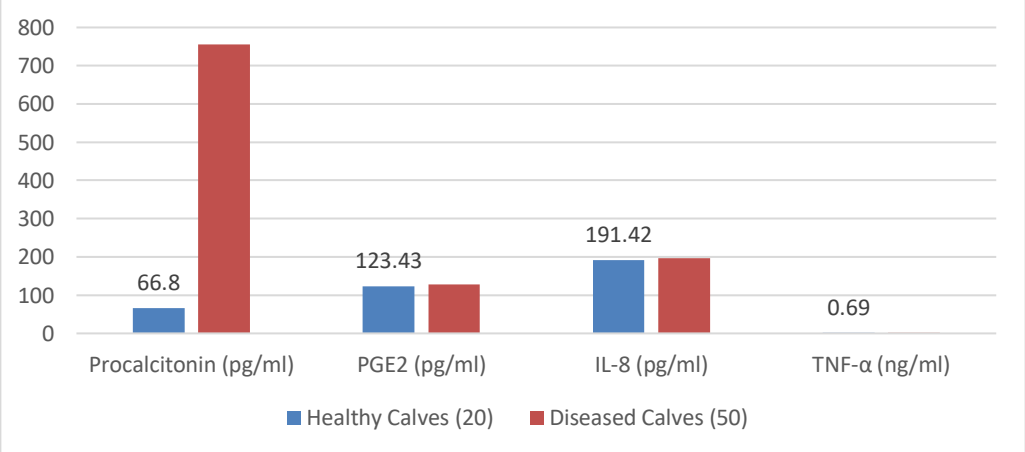


Figure 6. Procalcitonin and pro-inflammatory cytokines correlation.

DISCUSSION

Sepsis is more frequent in the first day after birth, manifested by diarrhea, depression, increased in respiratory and heart rate, dehydration, drowsiness and variant body temperature (Çitil and Gökçe, 2013). Mortality due to septicemia is common in large animals, early and accurate diagnosis is a challenge for veterinarians (House et al., 2015).

Sepsis is more frequent in the first days after birth, and Clinical signs appear due to sepsis, such as diarrhea, depression, increase in respiratory rate, increased heart rate, dehydration, inactivity, drowsiness, and body temperature variation (Çitil and Gökçe, 2013). Similar clinical signs were observed in this study, including diarrhea, depression, dehydration, increase in respiratory rate, decrease in the suckling reflex and hyperthermia in septic calves.

Fever and hyperthermia may develop in septic cases, as mentioned by (Yildiz *et al.* 2018).Also in the present study, some septic calves had variations in body temperature, but it appeared to be within the normal range, the body temperature of the septicemic calves may not be differ significantly than the healthy ones, but they are higher in their values and most of the previous literature revealed fever in septicemic conditions. Therefore no statistical difference in body temperature between septic and control calves.

Our study showed tachycardia and dehydration with increased respiratory rate in septicemic calves, the same findings were recorded by (Ercan *et al.* 2016). According to the causes of sepsis indicated by Naseri (2017), leukocytes may alter in a septic patient by increasing or decreasing. Our investigation found a significant difference in WBCs between control and septicemic calves, which is similar to (Akyüz and Gökce, 2016). Septicemia may be due to different etiological factors but, bacteria are still the most common cause of sepsis and *E. coli* is the most common in septic patients (Çitil and Gökçe, 2013), this is similar to our study but, it differs from other bacterial species *isolated; Klebsiella spp., B.Hemolytic streptococcus, staphylococcus aureus, and Pasteurella haemolytica*.

In this study, the blood culture diagnosis for sepsis had low sensitivity, and negative results must be cautiously interpreted as mentioned by Fecteau *et al.* (2009). Procalcitonin rises in acute inflammatory case, especially in bacterial infections, and is considered to be a septic–specific biomarker and high procalcitonin is considered a poor prognosis indicator; however, PCT rises in systemic bacterial infections but not in local infections (Hacımustafaoğlu, 2017). In the present study, there is a significant increase in procalcitonin in septicemic calves compared to healthy calves, similar to studies of (Rieger *et al.* 2014; Bonelli *et al.* 2018; Kirbas *et al.* 2019), which found an increase in serum PCT in humans, horses, dogs, and cows with bacterial septicemia.

In septicemic calves it has been reported for many times by various researchers that markers such as TNF-α, IL-8, PGE2, procalcitonin and neopterin which are revealing theresults of immune response during the infections could be used in observing the prognosis in infections,in this study, there were significant increases not only in pro-inflammatory cytokines (TNF-α and PGE2) but also in serum procalcitonin, similar to the study of Ercan *et al.* (2016), but there was a difference in serum procalcitonin increase level between the two studies, the level was higher in our study, serum PCT concentrations increased more than eleven times (~11) in calves with septicemia more than in healthy non-septicemic calves compared by four times (~4) increase in the calves with septicemic colibacillosis than in the healthy calves (Nazli *et al.*, 2016).

CONCLUSION

Serum PCT as a septic biomarker in calves; its measurements differentiate healthy from septic calves. It is necessary to conduct further research in order to determine whether procalcitonin can reduce the duration of the antimicrobial course and improve the prognosis for patients.

INTEREST IN CONFLICT DECLARATION

The authors have stated that they have no potential financial or personal conflicts of interest that could inappropriately influence the research, authorship, and publication of this article.

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