https://doi.org/10.17221/159/2017-VETMED

Evaluation of thrombomodulin and pentraxin-3 as diagnostic biomarkers in calves with sepsis

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ABSTRACT: Early diagnosis and treatment of sepsis in patients are crucial for their survival and can help reduce mortality rates. Novel biomarkers, such as thrombomodulin and pentraxin-3, have been used as diagnostic, prognostic and mortality indicators in patients with sepsis. Plasma thrombomodulin is a vascular endothelial membrane-bound glycoprotein and pentraxin-3 is an acute-phase protein. In the present study, thrombomodulin and pentraxin-3 levels were determined in calves with sepsis, to determine their diagnostic values as well as usefulness as indicators of health status. To this end, 20 neonatal calves with sepsis (G1) and ten healthy neonatal calves (G2) were used. Additionally, group G1 was also divided into two groups consisting of surviving (G1-S; n = 9) and non-surviving calves (G1-NS; n = 11). A single blood sample was collected from all the calves and the prepared serum samples were used to measure thrombomodulin and pentraxin-3 levels using bovine-specific ELISA kits. The serum concentrations of thrombomodulin and pentraxin-3 were found to be significantly higher ($P < 0.01$) in the G1 group than in G2. Thrombomodulin and pentraxin-3 levels were also found to be higher in the G1-NS group than in G1-S but the difference was not significant. We conclude that thrombomodulin and pentraxin-3 may have some diagnostic value in calves with sepsis. Furthermore, these findings may also help in understanding the pathogenesis of sepsis in neonatal calves. Further studies are required to determine the importance of thrombomodulin and pentraxin-3 as diagnostic and prognostic biomarkers in calves with sepsis and to evaluate the concentrations of these biomarkers also in other disease states.

Keywords: haematology; leucocytosis; biomarker; acute-phase protein; neonatal calves

List of abbreviations

CRP = C-reactive protein, **CRT** = capillary refill time, **DIC** = disseminated intravascular coagulation, **GRA** = granulocyte, **HCT** = haematocrit, **MAP** = mean arterial pressure, **PLT** = platelet, **PTX3** = pentraxin-3, **RBC** = red blood cell, **SEM** = standard error of the mean, **SpO_2** = peripheral oxygen saturation, **TLR** = toll-like receptor, **TM** = thrombomodulin, **WBC** = white blood cell

Sepsis is a condition defined by the development of a systemic inflammatory response syndrome (SIRS) as a result of confirmed or suspected infection and may be accompanied by systemic organ failure, often leading to death (Ok et al. 2015; Singer et al. 2016). Sepsis and diarrhoea are generally the most common causes of morbidity and mortality in neonatal calves that are related to the failure of colostral transfer. Sepsis can cause serious economic losses in farm animals (Fecteau et al. 1997; Basoglu et al. 2014). Even in humans, sepsis, which is often the most severe manifestation of acute infection, can cause multiple-organ failure and lead to death in 30–50% of cases. Thus, early diagnosis remains important in hospitalised patients with sepsis (Engel et al. 2007; Angus and Van der Poll 2013) for the initiation of evidence-based treatment and therapeutic interventions as

Supported by a grant received from Mehmet Akif Ersoy University, Scientific Research Project Office (Master of Science-Project No. 0393-YL-16).

https://doi.org/10.17221/159/2017-VETMED

required to increase the chances of survival (Levy et al. 2012; Nachimuthu and Haug 2012; Prucha 2015). Biomarkers, such as thrombomodulin (TM) (Hao and Wang 2013; Kim et al. 2016) and pentraxin-3 (PTX3) (Vanska et al. 2011; Uusitalo-Seppala et al. 2013), play an important role in both early diagnosis of the deseases and follow-up treatment (Christ-Crain and Muller 2007; Faix 2013).

Plasma TM is a vascular endothelial membrane-bound glycoprotein with a high affinity for thrombin and plays a major role in the protein C anticoagulant pathway. Plasma TM is produced primarily in the endothelial cells and, to a lesser extent, in lymphocytes, neutrophils, monocytes and dendritic cell subsets (Faust et al. 2001; Ciaramella et al. 2004). TM expression on the surface of endothelial cells is downregulated during inflammation, while a soluble form of TM is released into circulation resulting in an increased risk of thrombosis and disseminated intravascular coagulation (DIC) (Faust et al. 2001; Van de Wouwer et al. 2004; Sohn et al. 2005; Kim et al. 2016). PTX3 is an acute-phase protein similar in structure and function to C-reactive protein (CRP). The production of PTX3 is mediated by cytokine interleukin (IL)-1, tumour necrosis factor and toll-like receptor (TLR) agonists but not IL-6 and interferons. During inflammation, PTX3 is expressed in response to inflammatory signals and mainly produced at the site of inflammation by various cell types such as monocytes/macrophages, neutrophils, endothelial cells and dendritic cells (Mantovani et al. 2008; Savchenko et al. 2008). PTX3 has previously been characterised as a biomarker that is indicative of disease state and patient outcomes in certain infections and malignancies owing to its role in the immune system and inflammatory processes and relative ease of quantitation from blood (Latini et al. 2004; Bastrup-Birk et al. 2015). Since, unlike CRP, PTX3 is not affected by IL-6 levels, PTX3 levels in plasma reflect the severity of a direct infection and are thought to be less affected by other concurrent inflammatory conditions (Napoleone et al. 2004; Bottazzi et al. 2006; Savchenko et al. 2008). Novel biomarkers such as TM and PTX3 have been well-studied in human medicine but they have been relatively under-utilised in veterinary medicine. Furthermore, there is no reported study in calves with sepsis.

In this study, we tested the potential applicability of TM and PTX3 as biomarkers of systemic sepsis in neonatal calves.

MATERIAL AND METHODS

Animals. The animals used in this study consisted of 20 Holstein calves with sepsis with a history of diarrhoea (G1) and ten healthy Holstein calves (G2) that served as the control group. All calves ranged from one to 20 days old. Twenty calves with sepsis (G1) were divided into two groups consisting of surviving (G1-S; n = 9) and non-surviving (G1-NS; n = 11) animals. The clinical part of the study was carried out in a large animal clinic in the Faculty of Veterinary Medicine at Mehmet Akif Ersoy University, and all procedures were performed according to a study protocol approved by the Ethics Committee of the Veterinary Faculty, Mehmet Akif Ersoy University (Ethics Committee Certificate No. 2016/220).

Clinical examination. The inclusion criteria in the sepsis group included the lack of a suction reflex, lateral recumbency and weakness with at least two or more recorded SIRS signs (temperature > 39 °C or < 36 °C, heart rate < 100 or > 160 pulse/min, respiratory rate > 45/min, total leucocyte value > 12×10^3/µl or < 4×10^3/µl) plus infection or suspected infection (Fecteau et al. 1997; Thomas et al. 2004; Irmak et al. 2006; Radostits et al. 2007; Fecteau et al. 2009; Basoglu et al. 2014; Singer et al. 2016). Calves that were positive for systemic sepsis criteria were included in the study. The calves in the sepsis group were treated with the same standard therapy (intravenous fluids, antimicrobials and non-steroidal anti-inflammatory drugs) (Irmak et al. 2006; Radostits et al. 2007). The dehydration status was determined by skin elasticity (Radostits et al. 2007).

Blood sampling and laboratory analysis. Blood samples were collected through a catheter placed in the right jugular vein into tubes without anticoagulant (BD Vacutainer, UK) to determine the TM and PTX3 concentrations in serum and into tubes with K_3EDTA to determine haematological variables such as white blood cell (WBC), granulocyte (GRA), erythrocytes (RBC), haemoglobin (Hb), haematocrit (Ht) and platelet (PLT) values pre-treatment (0 hours). The samples were centrifuged (10 min at 4000 g) and the serum was stored at –20 °C until assayed. The serum concentrations of TM and PTX3 were determined using commercially available bovine-specific enzyme-linked immunosorbent assay ELISA kits according to the manufacturer's instructions (MyBioSource,

https://doi.org/10.17221/159/2017-VETMED

San Diego, USA). Absorption was recorded using a microplate reader (ELx800^{TM} Absorbance Microplate Reader, USA). The haematological variables were measured on an Abacus Junior Vet analyser (Diatron MI Ltd. Hungary) device within 15–30 min of sample collection. The granulocytes were counted together (neutrophils, eosinophils and basophils) in an automatic blood cell counter.

Statistical analysis. All data are presented as mean values and include standard error of the mean (mean ± SEM). The normality of data distribution was examined using the Kolmogorov-Smirnov test, which revealed that the data were normally distributed. Student's *t*-test and one-way ANOVA (with post-hoc Duncan's test) were used to compare the variables between groups. SPSS (version 14.01 for Windows, SPSS Inc, Chicago) was used to perform all statistical analyses. A *P*-value of < 0.05 was regarded as indicating statistically significant differences in concentrations.

RESULTS

The absence of suction reflex, hypothermia, coldness in the mouth or the extremities, weakness, tachypnoea, depression, prolonged capillary refill time and hyperaemic mucosa are the most common clinical signs of systemic sepsis observed in calves (Table 1). In this study, 11 calves did not survive and were assigned to group G1-NS while nine surviving calves were placed in group G1-S. Body temperature ($P < 0.01$), respiration rate ($P < 0.001$), oxygen saturation with pulse oximeter (SpO_2) ($P < 0.001$), capillary refill time (CRT) ($P < 0.001$) and heart rate ($P < 0.001$) showed significant differences between G1 and G2 (Table 1). The changes in haematological variables between the groups are shown in Table 2. The total WBC count and GRA count were found to be significantly higher ($P < 0.01$) in the G1 group than in G2 (Table 2). In G1, six calves presented with leucopenia, and 14 calves presented with leucocytosis. In the G1-NS group, three calves had severe leucopenia (< 3.5 × 10^3/μl), three calves had severe leucocytosis (> 33 × 10^3/μl) and the other five calves had moderate leucopenia or leucocytosis. Toxic changes of neutrophils were not assessed in this study. Monocytes and lymphocytes were also evaluated but there were no statistically significant differences between groups. Serum TM and PTX3 changes between the groups are shown in Table 3 and Table 4. The concentrations of TM and PTX3 were found to be significantly higher ($P < 0.01$) in G1 than in G2 (Table 3). Comparison of TM and PTX3 concentrations between groups G1-NS, G1-S and G2 revealed differences between G1-NS and G2. TM and PTX3 concentrations were also found to be higher in G1-NS and G1-S than in G1-S and G2, respectively, but the differences were not statistically significant (Table 4).

Table 1. Clinical findings in calves with sepsis and healthy calves (mean ± SEM)

Parameters	G1 (*n* = 20)	G2 (*n* = 10)	*P*-value
Temperature (°C)	37 ± 0.3	38 ± 0.8	0.008
MAP (mm Hg)	99 ± 8.1	114 ± 4.7	0.114
Respiration rate (min)	58 ± 5.6	28 ± 1.5	< 0.001
SpO_2 (%)	75 ± 1.7	94 ± 0.7	< 0.001
CRT (s)	6.7 ± 0.5	1.7 ± 0.1	< 0.001
Dehydration (%)	9.6 ± 0.4	–	–
Heart rate (min)	83 ± 3.4	121 ± 7.0	< 0.001

CRT = capillary refill time, G1 = calves with sepsis, G2 = healthy calves, MAP = mean arterial pressure, SpO_2 = pulse oximeter-oxygen saturation

Table 2. Haemogram parameters in calves with sepsis and healthy calves (mean ± SEM)

Parameters	G1(*n* = 20)	G2 (*n* = 10)	*P*-value
WBC (× 10^3/μl)	18 ± 2.8	8.9 ± 0.3	0.004
GRA (× 10^3/μl)	11 ± 2.1	3.2 ± 0.4	0.001
RBC (× 10^6/μl)	7.5 ± 0.4	7.7 ± 0.3	0.737
HCT (%)	25 ± 1.4	25 ± 0.8	0.748
PLT (× 10^3/μl)	484 ± 52	556 ± 47	0.316

G1 = calves with sepsis, G2 = healthy calves, GRA = granulocytes, HCT = haematocrit, PLT = platelets, RBC = red blood cells, WBC = white blood cells

Table 3. Thrombomodulin and pentraxin-3 levels in calves with sepsis and healthy calves (mean ± SEM)

Parameters	G1 (*n* = 20)	G2 (*n* = 10)	*P*-value
Thrombomodulin (ng/ml)	1.48 ± 0.19	0.77 ± 0.05	0.002
Pentraxin-3 (ng/ml)	3.31 ± 0.17	2.62 ± 0.15	0.007

G1 = calves with sepsis, G2 = healthy calves

https://doi.org/10.17221/159/2017-VETMED

Table 4. Thrombomodulin and pentraxin-3 concentrations in surviving and non-surviving calves with sepsis and healthy calves (mean ± SEM)

Parameters	G1-NS (n = 11)	G1-S (n = 9)	G2 (n = 10)
Thrombomodulin (ng/ml)	1.69 ± 0.28^{a}	1.21 ± 0.23^{ab}	0.77 ± 0.056^{b}
Pentraxin-3 (ng/ml)	3.39 ± 0.30^{a}	3.22 ± 0.14^{ab}	2.62 ± 0.15^{b}

Significant differences (P < 0.05) in values between G1-NS, G1-S and G2 are indicated by different letters in the same row
G1-NS = non-surviving calves with sepsis, G1-S = surviving calves with sepsis, G2 = healthy calves

DISCUSSION

Neonatal sepsis is one of the major health hazards in livestock rearing due to its high mortality rate (Aldridge et al. 1993). Clinical findings in the early stages of sepsis are not very specific but are still crucial to sepsis scoring and follow-up therapy (Radostits et al. 2007; Fecteau et al. 2009). Symptoms such as cold extremities, dehydration, weak pulse, prolonged capillary refill time, weakness, depression, dehydration, hypothermia and lateral recumbency have been reported in calves diagnosed with sepsis (Radostits et al. 2007; Fecteau et al. 2009). The symptoms of the calves with sepsis in the current study were similar to those reported in previous investigations (Aldridge et al. 1993; Fecteau et al. 2009; Basoglu et al. 2014).

Leucocytosis and left shift are common symptoms in sepsis and generally indicate poor prognosis when coupled with severe leucocytosis or leucopenia (Munford 2005; Irmak et al. 2006; Tornquist and Rigas 2010; Basoglu et al. 2014; Roland et al. 2014). In the present study, it was determined that individual total WBC counts of the calves in G1 were above or below the mean level of G2 and the mean level of G1 was significantly higher (P < 0.01) than that of G2. In the G1-NS group, three calves had severe leucopenia (< 3.5 × 10^3/µl) and three of them severe leukocytosis (> 33 × 10^3/µl). In ruminants, neutropenia occurs during the first one or two days of severe acute inflammatory conditions such as sepsis, and the number of neutrophils rarely exceeds 30 × 10^3/µl (Tornquist and Rigas 2010; Roland et al. 2014). Persisting neutropenia, severe persistent leucocytosis or a degenerative left shift indicate a guarded or poor prognosis (Roland et al. 2014). The results of this study show that a significant decrease or increase in total WBC counts may provide information about mortality when accompanied by clinical signs.

Currently available tests do not allow either early or specific identification of sepsis in patients; instead, diagnosis is often indirect and presumptive and is based on physical and laboratory findings, which may or may not be subject to change under septic conditions. Traditional markers such as WBC counts, body temperature and CRP concentrations are not reliable for assessing the severity of the disease and probable lethality. Novel biomarkers are now being used to diagnose and evaluate the risk of sepsis, as well as to monitor pathological changes during treatment periods, prognosis, the probability of lethal outcomes and to provide a preliminary view of possible clinical consequences (Christ-Crain and Muller 2007; Faix 2013; Basoglu et al. 2014). Numerous studies have been conducted so far on the role of biomarkers such as TM and PTX3 in human patients with sepsis but there is no report on livestock. The studies conducted on humans and laboratory animals have identified elevated TM concentrations in conjunction with disease processes that cause haemostatic disorders and endothelial damage (Ohdama et al. 1994; Wu et al. 2016; Lin et al. 2017). TM binds to high-mobility group box 1 protein and thereby prevents activation of leucocytes under *in vitro* conditions and lipopolysaccharide-induced lethality under *in vivo* conditions (Abeyama et al. 2005). Hao and Wang (2013) classified plasma TM concentrations in neonates with sepsis into the following groups based on probable clinical adverse reactions: extremely critical, critical and non-critical with values of 25.5, 17.3 and 13.3 µg/l, respectively; all these values were significantly higher than in the control group (9.8 µg/l). Sepsis is frequently complicated by endothelial cell injury. This is critical in the progression from DIC to multiple organ dysfunction syndrome (MODS) and subsequent mortality in septic patients. In patients with DIC, persistent thrombotic activity is closely linked to MODS and lethality and should be monitored by recording serum concentrations of TM (Lin et al. 2008). In patients with sepsis, the mean plasma concentration of TM is significantly higher in patients with MODS as a co-morbidity than in patients without MODS. Complications were also seen in human patients with plasma TM concentrations of above

https://doi.org/10.17221/159/2017-VETMED

6.0 ng/ml, suggesting that TM concentrations are a useful indicator for understanding MODS during sepsis (Iba et al. 1995). TM concentrations are also higher in lethal cases of sepsis than in surviving patients (Boldt et al. 1995; Lin et al. 2008). Serum thrombomodulin concentrations were found to be increased in different paediatric sepsis syndromes and were correlated with the severity and mortality of the disease (Lin et al. 2017). In the present study, the concentration of plasma TM in calves with sepsis was significantly higher than in the control group ($P < 0.01$) (Table 3). In addition, the TM concentrations of non-surviving calves with sepsis were found to be higher than those of surviving calves, although the differences were not significant (Table 4). While not specifically assessed in this study, we conclude that this observation may be related to the development of DIC and multiple-organ failure as previously reported in sepsis (Boldt et al. 1995; Iba et al. 1995; Lin et al. 2008; Ogawa et al. 2012; Hao and Wang 2013; Yasuda et al. 2016). A limitation of this study is that DIC and other specific parameters that indicate multiple organ failure were not evaluated. Comparative analysis of the aforementioned studies shows that TM may indeed be a useful marker for the detection of endothelial damage, the development and pathogenesis of DIC and multi-organ failure in calves with sepsis.

PTX3 is an inflammatory mediator produced by various cells in peripheral tissues as well as endothelial cells. During the early phase of inflammation, PTX3 recognises microbial moieties, activates the classical pathway of the complement system and facilitates antigen recognition by macrophages and dendritic cells (Garlanda et al. 2005). Evaluation of systemic PTX3 concentrations has indicated that non-survivors have consistently high PTX3 concentrations, which could represent a possible way to identify high-risk patients (Vanska et al. 2011). Consistently high levels of circulating PTX3 from the first day of sepsis onwards may be associated with mortality. PTX3 correlates with sepsis severity and coagulation/fibrinolysis dysfunction associated with sepsis (Mauri et al. 2010). The concentration of PTX3 in blood is usually low under normal conditions (< 2 ng/ml in humans) but increases rapidly during septic conditions, endotoxaemia and other SIRS events (Bottazzi et al. 2009). Uusitalo-Seppala et al. (2013) conducted a cohort-based study in which 537 emergency room human patients were divided into the following groups: group 1 (no SIRS, without bacterial infection), group 2 (no SIRS, with bacterial infection), group 3 (with SIRS, without bacterial infection), group 4 (with sepsis) and group 5 (with severe sepsis). The median PTX3 concentrations (2.6, 4.4, 5, 6.1 and 16.7 ng/ml, respectively) were significantly different between all groups. It is evident that median PTX3 concentrations were significantly higher in severe sepsis patients compared to others (16.7 vs 4.9 ng/ml) and in non-survivors compared to survivors (14.1 vs 5.1 ng/ml). Muller et al. (2001) also reported that in critically ill human patients, PTX3 concentrations are markedly elevated (SIRS patients; 28 ng/ml, septic shock; 250 ng/ml) compared with control donors (1.04 ng/ml). Hamed et al. (2017) determined the diagnostic cut-off values for PTX3 in human patients with sepsis (≥ 5.0 ng/ml) and septic shock (≥ 9.0 ng/ml). In the present study, PTX3 concentrations in calves with sepsis were also significantly higher ($P < 0.01$) than in the control group (Table 3). The elevated PTX3 concentrations in calves with sepsis demonstrate the potential usefulness of this acute-phase protein as a diagnostic marker. Furthermore, the higher concentrations of PTX3 in non-surviving calves, although not statistically significant, warrant future investigations for its use as a prognostic indicator. The findings of the present study suggest that PTX3 can be an important indicator in calves with sepsis, which is supported by previous studies (Muller et al. 2001; Mauri et al. 2010; Huttunen et al. 2011; Vanska et al. 2011; Uusitalo-Seppala et al. 2013; Hamed et al. 2017) conducted in human medicine.

In conclusion, our findings suggest that TM and PTX3 may hold significant diagnostic value for sepsis in neonatal calves. An increase in plasma TM concentrations may indicate the presence of endothelial damage and haemostatic disorders in calves, but further investigations with other endothelial and DIC markers are needed. We also showed that TM and PTX3 can be used to understand the pathogenesis of sepsis in calves and that elevated TM and PTX3 concentrations in non-surviving calves may hold some value as prognostic biomarkers. Further longitudinal studies are also required to investigate these possibilities. We believe that the findings of the present study can represent an important basis for future studies into these biomarkers in veterinary medicine. Further

https://doi.org/10.17221/159/2017-VETMED

investigations are needed to evaluate the concentrations of these biomarkers in other disease states.

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Received: December 7, 2017
Accepted after corrections: May 11, 2018