Original Article

Plasma Procalcitonin concentration in healthy calves and calves affected by septic Systemic Inflammatory Response Syndrome

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Abstract

Sepsis in calves still represents a big challenge for the veterinarians. Blood culture and clinical signs combined with a complete blood count have been used for the diagnosis of sepsis. Recent literature in humans and various animal species has been focused on sepsis-specific biomarkers, such as procalcitonin (PCT), that may represent a more accurately and efficiently source of sepsis diagnosis.

The aims of this study were to evaluate plasma PCT concentrations in healthy and septic calves. Twenty healthy control calves and 58 sick calves affected by septic Systemic Inflammatory Response Syndrome (SIRS) based on SIRS score and clinical findings were included. Septic SIRS calves were further divided in septic SIRS survivors (SSS) and non-survivors (SSNS). Plasma PCT concentrations were measured with a commercial ELISA assay for the bovine species. The receiver operating characteristic curve was used to determine cut-off values and corresponding sensitivity and specificity levels for the diagnosis of sepsis. Differences in plasma PCT concentration between groups (control *vs* SSS *vs* SSNS) have been evaluated.

Plasma PCT concentrations in healthy and septic SIRS calves were 33.3 pg/mL (0 - 44.3) and 166.5 pg/mL (85.9 - 233.0), respectively, with statistically significant differences ($P<0.001$) between groups. The calculated most accurate cut-off value to predict septic SIRS was 67.39 pg/mL (81.0% sensitivity, 95.0% specificity). Plasma PCT levels were 127.4 pg/mL (72.2 – 216.0) and 234.3 pg/mL (204.5 – 309.4) in the SSS and SSNS subgroups, respectively. Statistically significant differences were found among groups (control *vs* SSS and SSNS, $P<0.0001$; SSS *vs* SSNS, $P>0.05$). These results confirmed an increase in PCT concentration in calves with septic SIRS as previously reported in humans and other species.

Keywords: Calves; Diagnostic Test; Procalcitonin; Sepsis; Systemic Inflammatory Response Syndrome.

Introduction

Since the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference in 1991, the term *sepsis* has been defined as the "systemic inflammatory response to infection" (Bone et al., 1992). The expression "Systemic Inflammatory Response Syndrome" (SIRS) describes a clinical condition that represents the culmination of the activation of a complex network of acute endogenous mediators, which lead to an uncontrolled and widespread inflammation (Alberti et al., 2005). The SIRS can be associated with many different factors, including hypoxia, burns, trauma, immunologic reactions, bacterial and viral infections (Bone et al., 1992; Alberti et al., 2005). The confirmation of microbial infection in the presence of SIRS is required for a diagnosis of sepsis (Zabrecky et al., 2015).

Sepsis in calves has been reported to be sporadic or epidemic, reaching the 30% of prevalence if predisposing factors for the development of neonatal septicemia are present (Aldrige et al., 1993; Fecteau et al., 2009). Many factors predispose calves to sepsis such as failure of passive transfer, management deficits and adverse environmental conditions, cold stress, protein-energy malnutrition, micronutrient deficiencies and bacterial colonization of a local site, such as the umbilicus, gastro-intestinal tract or respiratory system (House et al., 2015). The pathophysiologic changes associated with this inflammatory activation due to sepsis are dehydration, alteration in heart and respiratory rates, body temperature, mucous membrane status and capillary refill time, leukopenia, hypotension and generalized weakness (House et al., 2015). The definitive diagnosis of sepsis is based on a blood culture. However, the sensitivity of blood culture might be low and a negative result must be interpreted with caution (Fecteau et al., 2009). In veterinary medicine, clinical signs combined with a complete blood count (CBC) and scores are considered useful in the diagnosis of sepsis. However, sepsis-specific biomarkers have become a recent focus of research both in human and in veterinary species because they may represent a more accurately and

efficiently source of sepsis diagnosis (Fecteau et al., 2009; Fielding and Magdesian, 2015; Ercan et al., 2016).

Procalcitonin (PCT) has been investigated as a biomarker of sepsis in humans (Riedel, 2012; Deliberato et al., 2013; Afsar and Sener, 2015), and different animal species such as equine (Toribio et al., 2003; Pusterla et al., 2006; Rieger et al., 2014; Bonelli et al., 2015a; Bonelli et al., 2015b; Barton et al., 2016; Bonelli et al., 2017), bovine (Ercan et al., 2016) and canine (Giunti et al., 2006; Yilimaz et al., 2008). Healthy individuals have very low serum PCT concentrations due to the restriction of the CALC-I gene transcription by the neuroendocrine cells in the thyroid gland and in the lung (Riedel, 2012; Afsar and Senar, 2015). The expression of the CALC-I gene is up-regulated in many tissues and cell types in septic men (Riedel, 2012; Afsar and Senar, 2015) and animals (Toribio et al., 2003; Giunti et al., 2010); thus, PCT is released into the circulation from many tissues and cell types in the body. In septic human patients, PCT rises rapidly (within 3 to 6 hours) and decreases by half within 24 hours when the infection and/or host immune response is controlled (Riedel, 2012; Afsar and Senar, 2015). Procalcitonin seems also to remain stable in blood specimens even at room temperature, unlike other markers of sepsis (Carrol et al., 2002). In veterinary medicine, some studies have been published concerning the evaluation of PCT in septic and not septic foals (Bonelli et al., 2015a), adult horses (Rieger et al., 2014; Bonelli et al., 2015b; Bonelli et al., 2017), and dogs (Giunti et al., 2006; Yilmaz et al., 2008). The aim of the present work was to evaluate the plasma PCT concentration in healthy and septic calves to verify differences.

Materials and Methods

Animals

The present *in vivo* multicentric experimental trial in clinical setting was approved by Italian Animal Care (DL 116/92) and approved by the Institutional Animal Care and Use Committee of the

University of Pisa (Approval number 2825; Approval date 28 January 2014) and University of Milan (Approval number: 2; Approval date 15 February 2016). Concerning Cornell University, blood samples were residual blood routinely collected from calves upon hospital admission for the purpose of immediate point of care chemistry testing. A written consent was obtained from the owners of all calves included. During the research period, a total of 260 calves were admitted to the veterinary teaching hospitals involved in the study, however, authors excluded cases that did not fit the inclusion criteria. In the present study, 78 calves aged 9.6±4.3 days were included. Twenty out of 78 were healthy Holstein calves (11/20 females and 9/20 males) aged 8.4±3.9 days from the university dairy farm of the University of Pisa. Fifty-eight out of 78 (35/58 females and 23/58 males), aged 10.6±4.2 days, were sick client-owned calves referred to three participating veterinary teaching hospitals providing secondary health care (the Veterinary Teaching Hospital "M. Modenato", University of Pisa, Italy; the "Nemo" Farm Animal Hospital, Cornell University, USA; the Clinic for Ruminants and Swine, University of Milan, Italy). Sick calves were Holstein Friesian (n=50/58) or belonged to mixed dairy breeds (n=8/58).

Collection of clinical and clinical pathological data

A complete history was recorded, when possible, for each calf at admission time, especially information concerning previous antimicrobials treatment. All calves were subjected to a complete physical examination and blood collection for CBC with differential and blood culture at admission time. Calves needed only manual restraint for all procedures. A 1-mL blood sample for CBC was collected from the jugular vein in a dipotassium salt of ethylenediaminetetra-acetic acid (K2EDTA) test tube (FL Medical) and analyzed by a cell counter (ProCyte Dx, IDEXX) within 5 minutes of collection. The K2EDTA was also used for a blood smear, which was air-dried and stained by an automatic stainer (Aerospray 7150 Hematology Slide Stain-Cytocentriguge). The differential cell count was performed by microscopic examination at 400 X and 1000 X magnification counting 100 cells. A sample for blood culture was collected aseptically and a commercial culture system

125 (OXOID SIGNAL Blood Culture System, Oxoid) was used as already reported (Daley et al., 1990;
126 Rohner et al., 1995). The outcome was recorded for all the sick client-owned calves as "discharged
127 *vs* died/euthanized".
128
129 Inclusion criteria
130 Calves less than 3 weeks old were enrolled in the present study. Each calf was scored
131 according to a SIRS scale used by others (Trefz et al., 2016). In order to be included in the septic
132 SIRS group, calves needed a positive SIRS score as inclusion criteria. SIRS positivity was based on
133 the presence of two or more of the following criteria (Trefz et al., 2016): presence of an abnormal
134 leucocyte count, *i.e.* leukopenia or leukocytosis (reference interval, 5–12 × 10^9/L); hyperthermia or
135 hypothermia (reference interval, 38.5– 39.5 °C); tachycardia (> 120 beats/min); and tachypnea (>
136 36 breaths/min). Calves with SIRS were considered septic if there was clinical or necropsy evidence
137 of septicemia, as reported by others (Lofstedt et al., 1999). In particular, the ante-mortem criteria
138 were: (1) positive blood culture, (2) culture of the same bacterial agent from ≥2 body fluids, or (3)
139 culture of a bacterial agent from a single joint in a calf with joint effusion involving multiple joints;
140 and the post-mortem criteria were: (1) morphologic changes such as multiple disseminated
141 abscesses of similar size, purulent vasculitis and intravascular identification of bacteria, or fibrin in
142 multiple body cavities; (2) bacterial isolation from heart blood; or (3) recovery of the same bacterial
143 organism from ≥2 body tissues (excluding intestine). Calves included in the control group must not
144 show neither signs of SIRS (SIRS score = 0), nor clinical signs of septicemia.
145
146 Evaluation of plasmatic PCT concentration
147 A 2.5-mL aliquot was collected at admission time in heparinized tubes (FL Medical) and
148 immediately centrifuged at 2.100 relative centrifugal force (RCF) for 10 minutes. The harvested
149 plasma was placed in sterile tubes, frozen at -18 °C, and analyzed for PCT in a single batch within 3
150 months. The concentrations of PCT were determined with a commercial kit for the bovine species

(Bovine Procalcitonin ELISA kit, MyBiosource.com). The intra-assay coefficient of variation was determined from 10 replicates of calf plasma samples with known low and high PCT concentrations. These samples were obtained by addition of standard PCT given to the ELISA kit manufactory in blank samples of calf blood. The inter-assay coefficient of variation was determined from values obtained by repeating the analysis of duplicate samples with low and high PCT concentrations in 5 different assays. Samples were measured in 10 replicates in a single assay and in 5 different assays. The intra- and inter-assay coefficient of variations were both < 15%, the limit of detection of the method was 10 pg/mL. To establish the detection limit for bovine plasma PCT, we performed repeated PCT measurement using bovine samples with low PCT concentrations (<10.0 pg/ml). Results less than LOD were expressed as 0.

Statistical analysis

A Kolmogorov-Smirnov test was applied to verify data distribution. Descriptive data are reported as median and percentile (25% and 75%) for both control and septic SIRS groups. A Mann-Whitney U test for unpaired data was performed to verify differences in plasma PCT concentration between the healthy controls and the septic SIRS groups. The receiver operating characteristic (ROC) analysis was performed to obtain specificity and sensitivity of the test at various cutoff values with a confidence interval of 95%. The likelihood ratio was calculated for each cut-off value. Animals included in the septic SIRS group were further divided in two subgroups, depending on the outcome of disease, *i.e.* whether they recovered (septic SIRS survivors subgroup – SSS) or euthanized or died (septic SIRS non-survivors subgroup – SSNS). A Kolmogorov-Smirnov test was applied to verify data distribution for PCT levels in the SSS and SSNS subgroups. The results did not show a Gaussian distribution for PCT levels in both the subgroups, so descriptive data are reported as median and percentile (25% and 75%). The Kruskal-Wallis test for unpaired data and Dunn's multiple comparisons post-hoc test were carried out to verify differences in plasma PCT concentration among groups (control *vs* SSS *vs* SSNS).

The significance level was set at $P < 0.05$. A commercial statistics software was used (GraphPad Prism 6).

Results

Collecting a complete history was not possible for every calf included. All 20 healthy calves used as a control group showed neither signs of SIRS (SIRS score = 0) nor clinical signs of septicemia, and all of them remained healthy for the entire study period. All 58 sick calves presented a positive SIRS score (≥2) and clinical or necropsy evidence of septicemia. The main complains of the sick calves referred were septicemia, pleuritis, pneumonia, diarrhea with signs of dehydration and electrolyte imbalance, peritonitis, omphalitis, polyarthritis. Positive blood cultures were used to diagnose 5 cases (8.6% of all blood cultures were positive). *E. coli* was cultured as single organism from all calves with positive blood culture. Culture of the same pathogen from ≥2 body fluids antemortem (e.g. peritoneal fluid, bronchoalveolar lavage fluid) were used to diagnose 45 cases: *E.coli* accounted for 30/45 (67%), others bacteria isolated were *Campylobacter* spp. (6), *Pasteurella haemolytica* (4), *Clostridium* spp. (3), *Enterococcus* spp. (2). A positive antemortem joint culture from calves with polyarthritis was used to diagnose septicemia in 2 cases. In both cases the isolated bacterial was *E. coli*. Regarding post mortem evaluation, morphologic lesions such as multiple abscesses, purulent vasculitis, intravascular identification of bacteria, and fibrin exudation in multiple body cavities, were present in 6 calves. All 58 sick calves satisfied the criteria for a diagnosis of septicemia, thus they were included in the septic SIRS group.

The plasma PCT concentration was 33.3 pg/ml (0 - 44.3) in the control group and 166.5 (85.9 - 233.0) in the septic SIRS group (Fig.1). The Mann-Whitney U test for unpaired data showed a statistical difference between the healthy and septic SIRS groups ($P<0.0001$). The resulting area

under the ROC curve was 0.92 (95% CI: 0.86 to 0.98, P< 0.001) (Fig. 2), and the best cut-off value for discriminate healthy calves from calves critically ill with clinical evidence of SIRS and septicemia was 67.39 pg/mL, with 81.0% mean sensitivity value (95% confidence interval 68%–90%), 95.0% mean specificity value (100% confidence interval 33% – 100%) (Table 1).

Fifty-two out of 58 (89.7%) septic SIRS calves recovered and they were included in the SSS subgroup; five out of 58 (8.6%) septic SIRS calves were euthanized for not economical reasons, and 1 out of 58 (1.7%) died, thus 6 out of 58 calves were included in the SSNS subgroup. The plasma PCT concentrations were 127.4 (72.2 – 216.0) pg/mL and 234.3 (204.5 – 309.4) pg/mL in the SSS subgroup and in the SSNS, respectively. The Kruskal-Wallis test for unpaired data indicated that there was a statistical significant difference for PCT (P < 0.001) between the three groups of calves (SSS, SSNS, control). The post-hoc Dunn's multiple comparisons test showed statistical differences between the control group *vs* SSS subgroup (P<0.0001), between control *vs* SSNS subgroup (P<0.0001) and between SSS *vs* SSNS subgroups (P<0.02).

Discussion

Despite the fact that neonatal sepsis is the third most common cause of mortality in large animal neonates, an early diagnosis remains a challenge for both the veterinarians and the farm managers (Fecteau et al., 1997; Lofstedt et al., 1999; House et al., 2015).

In the present study, the plasma PCT concentration was statistically lower in healthy than in septic SIRS calves. Our results confirmed that PCT increases during septic SIRS in calves as already reported in the equine, canine and human species (Yilmaz et al., 2008; Riedel, 2012; Rieger et al., 2014; Afsar and Senar, 2015, Bonelli et al., 2015a; Bonelli et al., 2015b; Bonelli et al., 2017). Moreover, the mean plasma PCT concentration both for healthy and septic SIRS calves were

similar to those reported in horses (Bonelli et al., 2015a; Bonelli et al., 2015b; Bonelli et al., 2017), lower than those reported in human newborns (Altunhan et al., 2011) and children (Lacour et al., 2001), and greater than in dogs (Yilimaz et al., 2008). The dissimilarities with humans and dogs could be related to the different assay used. The results obtained for the control group were similar to those reported for healthy calves by others (Ercan et al., 2014; Ercan et al., 2016). Recently, Ercan and colleagues evaluated some biomarkers for healthy cattle and investigated the diagnostic value of determining the serum levels of PCT and other markers (neopterin, tumor necrosis factor alpha, prostaglandin E2, malondialdehyde, interleukin 8 and IFN-γ) in neonatal calves diagnosed with septicemic colibacillosis (Ercan et al., 2016). The authors found that the serum PCT concentrations were four times higher in septicemic calves with colibacillosis than in healthy ones (Ercan et al., 2016). Plasma PCT values of septic SIRS calves obtained in this study were similar to those described by Ercan and colleagues (2016). In our study, we included a wider population of calves affected by SIRS and septicemia caused by different conditions, while Ercan and colleagues (2016) enrolled a population of calves affected only by septicemic colibacillosis.

Plasma PCT concentrations were statistically different between control group *vs* SSS and *vs* SSNS subgroups. Plasma PCT levels were statistically higher in SSNS calves compared to the SSS ones. This result might indicate a possible role of PCT in predicting unfavorable outcome in septic SIRS calves, as already reported in humans for sepsis, severe sepsis and septic shock (Huang et al., 2016; Ko et al., 2016; Liu et al., 2016; Poddar et al., 2016). The low number of negative outcome (SSNS subgroup) compared to the number of calves recovered (SSS subgroup) limits the conclusions concerning the outcome that can be drawn from the present study. The prognostic value of PCT will need to be addressed in future studies.

The cut-off value (67.39 pg/mL) obtained in this study is lower compared with findings reported for human adults (Riedel, 2012), neonates and children (Lacour et al., 2001; Altunhan et

al., 2011), but similar to foals (Bonelli et al., 2015a). To the best of authors' knowledge, no cut-off values have been reported in literature for calves or adult cattle.

The surviving rate resulted from the present study might be slightly high for a septic population. At admission time few calves presented bacteremia, while the majority of the population had findings of localized sepsis. Even if the history collection was not possible for all cases, some of the calves included had already received treatment at home. This might explain the low number of "died/euthanized" calves.

A limit of the present study would be the low number of blood culture positive calves. A negative blood culture result must be interpreted with caution because many factors may interfere with bacterial isolation from a blood culture. Prior antibiotic therapy, presence of opsonizing antibodies, numbers of circulating bacteria, a relatively low volume of blood for culture and course of the diseases have been described as possible factors affecting the sensitivity of blood culture (Wilson and Madigan 1989; Fecteau et al., 2009). In the present study, the history that had been collected concerning previous antimicrobials treatment was incomplete for some subjects. Also, the blood culture was always performed at the admission and might be possible that not all calves were bacteremic at this moment. An improvement in history collection might be strongly considered for further studies.

Conclusions

Procalcitonin measurements allow to differentiate between healthy calves and critically ill calves with clinical evidence of SIRS and septicemia. Further studies could be carried out to investigate the role of PCT in shortening antimicrobial therapy, as already proposed in humane medicine. Moreover, recording the length of a completely recovery, not only the final outcome, might be an aim for further studies about PCT levels and survival analysis. Finally, PCT testing

should be also done in calves with non-septic SIRS for a better understanding of role of this biomarker in the diagnosis of SIRS.

Conflict of interest statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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426 **Table 1.**

427 Receiver Operating Characteristic (ROC) curve analysis data as assessed for the definition of a best
428 cut-off value for plasmatic Procalcitonin (PCT) concentration for discriminate healthy calves from
429 calves critically ill with clinical evidence of Systemic Inflammatory Response Syndrome (SIRS) and
430 septicemia (n=78).

431

Cut-off (pg/mL)	Sensitivity % (95% CI)[a]	Specificity % (95% CI)	Likelihood ratio
> 27.72	62 (35-85)	97 (85-100)	21.87
> 32.82	69 (41-89)	97 (85-100)	24.06
> 38.75	75 (48-93)	97 (85-100)	26.25
> 55.60	81 (54-96)	97 (85-100)	28.44
> 64.00	81 (69-90)	95 (73-100)	16.20
> 67.39	81.03 (68-90)	100.0 (83-100)	-
> 70.09	81.03 (66-88)	100.0 (83-100)	-

432

433 [a]95% CI, 95% Confidence interval

434 **Figure legends**

435

436 Fig. 1. Box and whisker plot showing median and 10-90 percentiles of Procalcitonin (PCT)
437 concentration levels in the septic Systemic Inflammatory Response Syndrome (SIRS) group (n=58
438 calves) and in the control group (n=20 calves). *** P<0.0001.

439

440 Fig. 2. Receiver Operating Characteristic (ROC) curve for the defined septic Systemic Inflammatory
441 Response Syndrome (SIRS) analysis performed to obtain specificity and sensitivity of the test at
442 various cut-off values with a confidence interval of 95%.

443