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(Article begins on next page)
SEPSIS AND BACTERIAL SUPPURATIVE MENINGITIS-MENINGOENCEPHALITIS IN CRITICALLY ILL NEONATAL PIEDMONTESI CALVES: CLINICAL APPROACH AND LABORATORY FINDINGS


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SUMMARY

Sepsis (S) and bacterial suppurative meningitis-meningoencephalitis (M-ME) are common causes of death in bovine neonates. The aim of this prospective study was to evaluate the prevalence of S and M-ME in critically ill neonatal Piedmontese calves. Critically ill animals up to 15 days old referred by practitioners were registered according to their status and subsequently assigned to clinical standardized score. Calves with a clinical score ≥ 5 were further assessed under a clinical and clinical-pathological protocol to strengthen the suspicion of S and M-ME. Critically ill neonatal calves sent for necropsy were included in the study as well. Fifty-nine calves were investigated, 26 of which referred alive and 33 dead. Ten out of the 26 clinically evaluated calves were classified as suspicious of S on the basis of the clinical and clinical-pathological protocols. S was confirmed by positive bacteriologic culture in 7 cases and in 3 cases on the basis of necroptic lesions. Concomitant suppurative M-ME suspected in 6 of these 10 calves was subsequently confirmed by CSF analysis or histological findings. Of the 33 calves examined only post-mortem, 20 showed pathognomonic findings of S and 14 signs of M-ME. The prevalence of S and M-ME was 46 and 36%, respectively. Clinical signs of S were confirmed to be vague and overlapping with other diseases. The developed protocol was highly accurate in predicting S in these neonatal calves.

KEYWORDS: bovine, neonatal diseases, sepsis, meningitis, meningocerephalitis, clinical signs.

Sepsis und bakterielle eitrige Meningitis-Meningoencephalitis bei kritisch erkrankten neugeborenen Kälbern der Piemonteser Rasse: Klinische Studie und Ergebnisse der Laboruntersuchungen

Sepsis (S) und bakterielle eitrige Meningitis-Meningoencephalitis (M-ME) sind häufige Ursachen, die zum Tod von neugeborenen Kälbern führen. Ziel der Untersuchung war es, die Prävalenz von S und M-ME bei kritisch erkrankten neugeborenen Kälbern der

Schlüsselwörter: Kalb Krankheiten bei Neugeborenen, Sepsis, Meningitis-Meningoencephalitis, klinische Symptome

INTRODUCTION: Sepsis (S), a major health problem in calves less than 2 weeks of age associated with a high mortality rate (Aldridge et al., 1993; Fecteau et al., 2009), is described as the systemic inflammatory response syndrome (SIRS) to an active infectious process (Bone et al., 1992; Levy et al., 2003; Goldstein et al., 2005). Decreased or failed passive transfer (FPT) of immunity and exposure to virulent pathogens are important risk factors for the development of S. The most frequent etiological agent is Escherichia coli (Lofstedt et al., 1999; House and Gunn, 2009; Fecteau et al., 2009), followed by Salmonella spp., Campylobacter spp., Kebisiella spp, Staphylococcus spp., and Streptococcus spp. (Seimiya et al., 1992; Fecteau et al., 2009). S usually involves multiple organs and most commonly affects the respiratory and gastrointestinal systems with rapid and often fatal progression of disease. Early clinical signs are vague and nonspecific (Fecteau et al., 2009; Vaala et al., 2009) and laboratory values may be altered (Lofstedt et al., 1999; Irmak et al., 2006). Since blood culture has a low sensitivity and results are available only after 24-72 h, scoring schemes have been developed for several species to help identify septic subjects early in the course of the disease and to improve favorable outcome of therapy (Brewer and Koterba, 1988; Fecteau et al., 1997a; Lofstedt et al., 1999; Moore et al., 2009). Neonatal bacterial suppurative meningitis-meningoencephalitis (M-ME) is commonly a result of S (Fecteau and George, 2004; House and Gunn, 2009). Bacteria can spread hematogenously to the leptomeninges, although the mechanism of pathogen entry is not clearly understood (Kim, 2003; Zachary, 2007; Fecteau et al., 2009). The definitive
diagnosis relies on abnormal findings of cerebrospinal fluid (CSF) which, in acute stages, shows elevated neutrophils and increased protein content and/or intracellular bacteria (Green and Smith, 1992; Scott and Penny, 1993; Fecteau et al., 2009; Mayhew, 2009a; Stokol et al., 2009). The aim of this prospective study was to evaluate and refine a protocol for diagnosis of S and bacterial M-ME in young Piedmontese calves. The proportion of S and bacterial M-ME was also investigated in the referred population.

ANIMALS, MATERIAL AND METHODS

ANIMALS
A total of 59 critically ill Piedmontese calves up to 15 days referred live or dead to the Teaching Hospital of the Faculty of Veterinary Medicine of Turin, between February 2008 and March 2010, were used for our study. All animals underwent complete physical and neurologic examination. A total clinical score, based on the classification scheme of Fecteau et al. (1997a) made up of five individual scores (attitude, hydration, feces, navel and sclera vessels), was assigned to each animal. Detailed anamnestic information of the dam and the calf were collected.

DEFINITION OF DISEASES
Calves were classified as suspicious of S if the clinical score was ≥ 5 and met at least one of the following criteria: presence of a focal infection site other than enteritis; serum glucose < 3.3 mmol/L; blood smear suggestive of S (left shift of neutrophils and signs of toxicity in neutrophils). S was confirmed by positive blood culture (in vivo) and/or bacterial isolation from tissue samples (post-mortem), along with evidence of macro- and microscopic lesions strongly suggestive of S. Calves were considered suspicious of M-ME if the neurologic examination was suggestive of an intracranial localization and CSF total cell count showed pleocytosis (> 10 cells/µL). M-ME was confirmed when the CSF analysis showed a neutrophilic pleocytosis and increased proteins (> 40 mg/dl) and/or when histological findings were indicative of the disease (Zachary, 2007). Calves with a clinical score < 5 were managed with specific investigation to diagnose the underlying disease. Calves with a clinical score ≥ 5 were included in the protocol described below. For critically ill Piedmontese calves up to 15 days of age sent for necropsy, a standardized data collection form was compiled by interviewing the referring veterinarian. A control group of 20 healthy Piedmontese calves, 1-15 days old, was included in the study. All animals were examined simultaneously by a board certified person in neurology and bovine health management (ADA). The study was performed according to ethical recommendations, animal welfare considerations and regulations and approved by the Health Direction Section of Piedmont Region (reference number 466/2008).
PROTOCOL FOR ASSESSMENT OF CALVES WITH A SCOTE ≥ 5

Blood for hematological and serum analyses was collected in EDTA-containing and plain tubes, respectively. Samples for blood gas analysis (pH, bicarbonate, Begeol, electrolytes (Na+, Cl-, iCa2++, K+), glucose, hemoglobin (Hb) concentrations and hematocrit (Hct) were anaerobically collected in a 2.5-mL heparinized plastic syringe (Preza-Pak II, Terumo) and immediately determined (i-STAT Analyzer, i-STAT Corporation). Blood samples for Celite Activate Clotting Time measurement (CELTIEACT - Abbot Point of Care Inc) were drawn into a plastic syringe and immediately dispensed into the sample well of a cartridge. For blood culture, a volume of 10 mL of blood was aseptically collected, added to a proper broth (Signal Blood Culture System, Oxoid Limited) and submitted for bacteria culturing and isolation.

A CSF sample was collected from the lumbosacral site from all calves showing neurologic signs, and stored at 4°C. Total nucleated cell count was assessed in the field using a Nageotte hemacytometer. For CSF differential cell count and total microprotein concentration, samples were analyzed within 1 h of collection in the lab. Blood culture and organism identification were performed using standard procedures.

Hematology (ADVIA120 Hematology System, Siemens Diagnostics), blood chemistry analysis including ALP, AST, CK, BUN, CREA, total bilirubin, R and Mg (ILab 300 Plus, Instrumentation Laboratories), and standard clotting profile composed of aPTT, PT and fibrinogen (Start 4 Hemostasis Analyzer, Diagnostica Stago) were performed in the lab. For left shift of neutrophils and toxicity, a blood smear was stained with May-Grünewald Giemsa and examined. Passive transfer of immunity was indirectly estimated in total serum solids by a temperature compensated hand-held refractometer (Leica VET 360 Total Strlids Refractometer, Leica Inc.).

POST-MORTEM EXAMINATION AND BACTERIOLOGICAL ISOLATION

Calves that did not survive or were humanely euthanized were sent for post-mortem examination.

Complete necropsy was performed. The internal organs and brain of all animals were collected and routinely processed for histopathological examination. Aerobic and anaerobic culture of tissue samples was performed.

STATISTICAL ANALYSIS

Statistical analysis was performed using a freeware statistical software package (R 1.12.2; http://www.r-project.org/). Normally distributed data were tested using the Shapiro-Wilk Normality Test. The analytical parameters (5th- 95th percentiles) of the control group were used as internal comparison data. The analytical parameters of the septic calves (median values) out of the 5th – 95th percentiles obtained from the control group were tested using the Wilcoxon signed-rank test. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the clinical and clinical-pathological methods used to suspect S were calculated. Results were considered significant if the P-value was less than 0.05.

RESULTS

Fifty-nine critically ill calves (29 males and 30 females; mean age, 9.6 ± 5.4 days) were investigated, 26 (44%) of which were clinically evaluated and 33 (56%) were examined only post-mortem.

CRITICALLY ILL CALVES

S was suspected in 10/26 (38%) calves with a score ≥ 5 and confirmed in 7/10 animals by positive blood culture and/or tissue isolation. In the 3 remaining animals, macro- and microscopic lesions were strongly suggestive of S (Tab. 1). Analytical parameters between the septic and control groups are listed in Table 2. In 5 animals a failure of
passive transfer of immunity was determined on the basis of decreased total serum solids (≤50 g/L). Table 3 shows the accuracy of clinical and clinical-pathological methods used to suspect S. M-ME was clinically suspected in 6 but confirmed in 7/10 calves with a score ≥5 (Tab. 1). The median number of CSF nucleated cells in suspicious M-ME calves was 92.3 cells/µl (39.7–1840.0); the mean CSF protein concentration was 151.4 + 122.8 mg/dl. The 16 non septic animals (score < 5) included calves with neonatal diarrhea (n = 7), otitis media/interna (n = 3), spinal trauma (n = 2), acute perinatal asphyxia (n = 2), arthritis (n = 1) and abomasal ulceration (n = 1).

CRITICALLY ILL REPORTED DEAD CALVES
Twenty (61%) out of the 33 dead calves were confirmed as septic on the basis of tissue bacterial isolation; 3 additional animals showed macro- and microscopic lesions strongly suggestive of S. Fourteen out of the 20 (70%) septic calves showed concurrent histological features of M-ME. The frequency of the most common signs in the septic and M-ME animals is reported in Figure 1; some of the calves showed more than one sign. Neonatal diarrhea (n = 8) and bronchopneumonia (n = 1) were diagnosed in 9 critically ill calves with negative tissue bacterial isolation but without gross and histopathological findings of S and/or M-ME. The cause of death in one other case was undetermined, but S and M-ME were excluded. E. coli accounted for 19 isolates (one of which yielded E. coli and Streptococcus bovis). Streptococcus pneumonia was isolated in only one subject. The prevalence of S and M-ME in the critically ill calves was 46% and 36%, respectively.

DISCUSSION
The reason to choose the Piedmontese breed was motivated by its economic relevance in Italy. Unweaned calves are very valuable and early diagnosis and treatment of neonatal infectious disease should be mandatory for a favorable outcome and cost reduction. Among infectious diseases, S accounts for considerable morbidity and mortality in bovine neonates (Fecteau et al., 2009; Vaala et al., 2009). To the best of our knowledge, this is the first report to evaluate the proportion and clinical features of S and M-ME in Italy and in the Piedmontese Breed. The high prevalence of S (46%) in this study is similar to that (51%) reported by Mosher et al. (1968) but higher than that found by other authors (Fecteau et al., 1997b; Lofstedt et al. 1999). Aldridge et al. (1993) reported an incidence of 26% in dairy and beef critically ill calves considering criteria similar to those we applied in our study. Clinical signs of S in this study were nonspecific, confirming that early diagnosis remains a challenge for the veterinarian and that clinical signs are likely to be attributed to other diseases (Fecteau et al., 2009; Vaala et al., 2009). Hematological and biochemical changes as described in our study, are both a consequence of systemic inflammation and evidence of organ dysfunction; our observation are in line with previous data (Aldridge et al., 1993; Gerros et al., 1995; Lofstedt et al., 1999; Irmak et al., 2006). The CELITE ACT test measures the time required for complete activation of the coagulation cascade (Hattersley 1, 966). The CELITE ACT reference values of the present study were comparable to those of a previous one (Riley and Lassen, 1979). In the present report, the CELITE ACT was used assuming that the bleeding disorders resulting from S could be identified in the field. Significant differences between septic and healthy animals were found; however, more extensive research of critically ill calves is required to validate the clinical reliability of this test. Calves with FTP have a high risk for developing neonatal S and subsequent M-ME (Fecteau et al., 2009). In the present study, 5 septic live animals showed a FTP on the basis of low total serum protein concentration, which may have been underestimated since dehydration may have contributed to an individual increase in total protein (Constable et
al., 1998). On the other hand, as it is customary for the Piedmontese breed to leave calves uncontrolled with the dam and allow them to suckle naturally erratic nursing attempts in contaminated areas (tail, hock) may have exposed the calves to a massive pathogen load, regardless of their immune status. *Escherichia coli* was confirmed to be the most frequent agent isolated from blood and tissue cultures from septic calves (Raska et al., 1978; Hariharan et al., 1992; Aldridge et al., 1993; Fecteau et al., 1997b; Lofstedt et al., 1999). *Streptococcus* spp. and *Staphylococcus* spp. cultured in 2 calves and one calf, respectively, have rarely been reported as a cause of S and M-ME (Seimiya et al., 1992; Fecteau et al., 1997b; Lofstedt et al., 1999). In 6 critically ill calves, although S was strongly suspected at necropsy, bacteriological culture of tissue specimens was negative. This could be related to prior antibiotic therapy, number of bacteria, and course of the disease. The clinical and clinical-pathological protocols developed in this study showed a sensitivity and specificity superior to those previously described (Fecteau et al., 1997a; Lofstedt et al., 1999). Since a score of 5 or more can be achieved with diseases other than S (i.e. severe acidosis and dehydration), the score was modified by also using the presence of hypoglycemia and/or alterations in neutrophils. With the use of these protocols we were able to identify septic calves earlier than before and to exclude the disease in a large number of animals thus limiting costly antimicrobial and supportive therapies. Furthermore, the sensitivity value of the protocols (lower 95% confidence interval: 59%) was probably underestimated due to the 3 calves in which bacteriological isolation was negative. The fact that all 3 had M-ME also reinforces the suspicion that they could actually have had S. A limitation to this study is the small sample size on which we were able to apply the clinical laboratory protocols. Their use on a larger population of critically ill calves is needed to confirm the promising results. M-ME is commonly secondary to S (Green and Smith, 1992; Fecteau et al., 2009). The overall prevalence of M-ME in the present study (36%) is similar to that (43%) reported by Mosher et al. (1968) in 103 necropsied calves. In other studies (Green and Smith, 1992; Stokol et al. 2009)), the prevalence was 2% and 18%, respectively. All animals with M-ME had concomitant S. The clinical signs in the 7 calves that underwent a complete neurological examination were only partly similar to those previously described. No hyperesthesia or exaggerated spinal reflexes were observed (Green and Smith, 1992; Scott and Penny, 1993; Fecteau and George, 2004; Fecteau et al., 2009). Trismus- detected in 3 animals- has rarely been reported (Jamison and Prescott, 1987), while gait abnormalities, proprioceptive and cranial nerves deficits are a confirmation of neuroparenchymal involvement. Neurologic signs, reported by the referring veterinarian in septic calves without M-ME lesions could be related to extreme terminal weakness, electrolyte and acid-base disturbances, hypoglycemia, hypothermia and pain (Scott and Penny, 1993; House and Gunn, 2009; Moore, 2009).

A neutrophilic pleocytosis was detected in all except one calf with clinical signs of M-ME in which CNS involvement was confirmed at necropsy. A previous steroid therapy could explain the normal CSF in this case. In conclusion, failure of passive antibody transfer remains an important risk factor for neonatal S and subsequent M-ME. Clinical signs of M-ME were mostly indicative of brain parenchymal involvement. Clinical signs of S were confirmed to be vague and overlapping with other diseases. The in field application of the clinical and clinical-pathological protocols was found to be highly sensitive and specific in predicting S in these neonate calves.

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REFERENCES


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Tables and Figures legend

Table 1: Critically ill septic calves: parameters for classifying animals with suspected S, neurologic evaluation, CSF, blood culture, outcome, post-mortem examination. *Calves pre-treated with antibiotics; ^a Central nervous system; ^b Blood smear suggestive of S; ^c Neutrophilic pleocytosis; ^d Pathological findings strongly suggestive of S; ^e Pathological findings of M-ME; ^f Not performed; ^g Blood smear not suggestive of S.

Table 2: Analytical parameters of septic calves outside of reference data and significantly different from control animals.

Table 3: Accuracy of clinical and clinical-pathological methods used to suspect S (N=26).

^a Denotes 95% confidence interval; ^b Positive predictive value; ^c Negative predictive value.

Figure 1: Frequency of the most common signs in the referred dead calves with S and M-ME.
### Table 1:

<table>
<thead>
<tr>
<th>Case</th>
<th>Total score</th>
<th>Focal infection</th>
<th>Serum glucose &lt; 3.3 mmol/L</th>
<th>Blood smear</th>
<th>Neurologic evaluation</th>
<th>CSF</th>
<th>Blood culture</th>
<th>Outcome</th>
<th>Necropsy/ Tissue Isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>5</td>
<td>CNS$^a$</td>
<td>No</td>
<td>+</td>
<td>Altered mental status, wide base stance, proprioceptive deficits, trismus, seizures</td>
<td>npl$^c$</td>
<td>E. coli</td>
<td>Deceased or euthanized</td>
<td>S$^d$/M-ME$^e$ E. coli</td>
</tr>
<tr>
<td>b*</td>
<td>5</td>
<td>Broncho pneumonia</td>
<td>No</td>
<td>+</td>
<td>Normal</td>
<td>X$^f$</td>
<td>Coagulase-negative Staphylococci</td>
<td>Survived</td>
<td></td>
</tr>
<tr>
<td>c*</td>
<td>6</td>
<td>CNS</td>
<td>No</td>
<td>-</td>
<td>Altered mental status, opisthotonus, tetraparesis, proprioceptive deficits, ventral strabismus, positional nystagmus</td>
<td>npl</td>
<td>Negative</td>
<td>Deceased or euthanized</td>
<td>S/M-ME E. coli</td>
</tr>
<tr>
<td>d</td>
<td>5</td>
<td>Not detected</td>
<td>No</td>
<td>+</td>
<td>Normal</td>
<td>X</td>
<td>E. coli</td>
<td>Deceased or euthanized</td>
<td>Not performed</td>
</tr>
<tr>
<td>e*</td>
<td>6</td>
<td>Broncho pneumonia</td>
<td>No</td>
<td>+</td>
<td>Altered mental status, wide base stance, proprioceptive deficits</td>
<td>Normal</td>
<td>Negative</td>
<td>Deceased or euthanized</td>
<td>S/M-ME E. coli</td>
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<tr>
<td>f*</td>
<td>5</td>
<td>CNS</td>
<td>No</td>
<td>+</td>
<td>Altered mental status, wide base stance, opisthotonus, proprioceptive deficits, trismus, positional nystagmus</td>
<td>npl</td>
<td>Negative</td>
<td>Deceased or Euthanized</td>
<td>S/M-ME</td>
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<tr>
<td>g*</td>
<td>7</td>
<td>Uveitis and CNS</td>
<td>Yes</td>
<td>-</td>
<td>Altered mental status, circling, head pressing, wide base stance, tetraparesis, proprioceptive deficits, trismus</td>
<td>npl</td>
<td>E. coli</td>
<td>Deceased or Euthanized</td>
<td>S/M-ME E. coli</td>
</tr>
<tr>
<td>h</td>
<td>11</td>
<td>Uveitis and CNS</td>
<td>Yes</td>
<td>-</td>
<td>Altered mental status, tetraparesis, proprioceptive deficits, nystagmus</td>
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<td>Negative</td>
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<td>S/M-ME</td>
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<tr>
<td>i</td>
<td>5</td>
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<td>No</td>
<td>+</td>
<td>Normal</td>
<td>X</td>
<td>E. coli</td>
<td>Survived</td>
<td></td>
</tr>
<tr>
<td>l</td>
<td>8</td>
<td>CNS</td>
<td>Yes</td>
<td>+</td>
<td>Altered mental status, tetraparesis, proprioceptive deficits</td>
<td>npl</td>
<td>Negative</td>
<td>Deceased or Euthanized</td>
<td>S/M-ME</td>
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### Table 2:

<table>
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<tr>
<th>Analytes</th>
<th>Units</th>
<th>Septic Calves</th>
<th>Reference data from control group (5th - 95th percentiles)</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td>Red blood cells (RBC)</td>
<td>x 10^{12}/L</td>
<td>8.93</td>
<td>5.45 – 8.80</td>
<td>0.033</td>
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<tr>
<td>Total serum protein</td>
<td>g/L</td>
<td>45</td>
<td>50 – 71</td>
<td>0.003</td>
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<tr>
<td>Serum glucose</td>
<td>mmol/L</td>
<td>3.8</td>
<td>4.4 – 6.5</td>
<td>0.009</td>
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<td>Urea</td>
<td>mmol/L</td>
<td>60.0</td>
<td>3.2 – 11.4</td>
<td>&lt; 0.001</td>
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<tr>
<td>Creatinine</td>
<td>μmol/L</td>
<td>350</td>
<td>107 – 190</td>
<td>0.04</td>
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<tr>
<td>Alkaline phosphatase (ALP)</td>
<td>UI</td>
<td>157</td>
<td>184 - 458</td>
<td>0.005</td>
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<tr>
<td>Aspartate amino transferase (AST)</td>
<td>UI</td>
<td>84</td>
<td>19 - 65</td>
<td>0.003</td>
</tr>
<tr>
<td>Creatine kinase (CK)</td>
<td>UI</td>
<td>840</td>
<td>41 - 284</td>
<td>&lt; 0.001</td>
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<tr>
<td>Sodium</td>
<td>mmol/L</td>
<td>142</td>
<td>133 - 138</td>
<td>0.01</td>
</tr>
<tr>
<td>Platelets</td>
<td>x 10^{3}/μL</td>
<td>326</td>
<td>485 - 1218</td>
<td>&lt; 0.003</td>
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<tr>
<td>Activated clotting time (ACT)</td>
<td>s</td>
<td>237</td>
<td>112 - 190</td>
<td>0.002</td>
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<tr>
<td>Fibrinogen</td>
<td>g/L</td>
<td>7.8</td>
<td>2.7 – 5.4</td>
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<tr>
<td>Prothrombin time (PT)</td>
<td>s</td>
<td>38</td>
<td>18 – 25</td>
<td>&lt; 0.001</td>
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### Table 3:

<table>
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<td>Sensitivity</td>
<td>100%</td>
</tr>
<tr>
<td>Specificity</td>
<td>84.2%</td>
</tr>
<tr>
<td><strong>PPV</strong> b</td>
<td>70%</td>
</tr>
<tr>
<td><strong>NPV</strong> c</td>
<td>100%</td>
</tr>
</tbody>
</table>

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*Table 2 and Table 3 data are presented in a tabular format with analytes, units, medians, minimums, maximums, and p-values.*
Figure 1:

NUMBER OF CALVES

*Neurologic signs included: altered mental status, opistotonus, recumbency, tremor, ataxia, circling

1.