Università degli Studi di Milano

GRADUATE SCHOOL OF VETERINARY SCIENCE
FOR ANIMAL HEALTH AND FOOD SAFETY

Doctoral Program in Veterinary Clinical Science

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Impact of colostral administration practices on the outcome of diarrhoeic calves and future therapeutic options for the failure of passive transfer of immunity

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Academic Year: 2013-2014

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A dissertation presented to the Graduate School of Veterinary Science for
Animal Health and Food Safety

by

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Chapter one

Introduction and review of literature

1.1 Introduction and objectives

Neonatal calf diarrhoea (NCD) is one of the most important problems in calf rearing in dairy farms worldwide. The U.S. National Animal Health Monitoring System reported that 57% of weaning calf mortality is dued to diarrhoea and most cases occurred in calves less than 1 month old (USDA, 2007). In Europe NCD is the most important disease problem in calves and causes a great economic loss (Østerås et al., 2007; Torsein et al., 2011). Diarrhoea is a complex, multifactorial disease with numerous infectious and noninfectious factors. Although different studies have aimed to identify risk factors for the presence of NCD in farms (Parè et al., 1993; Bendali et al., 1999; Berge et al.,2009; Pithua et al., 2009; Klein-Jöbstl et al., 2014), little is known about factors involved in the outcome in affected calves. Indeed, only few investigations are available in calves on the influence of some biochemical data on outcome of neonatal diarrhoea (Fayet and Overwater, 1978; Klee et al., 1979; Lorenz, 2004a; Lorenz and Vogt, 2006; Seifi et al., 2006). When adequate amounts of immunoglobulins (IgG) are not absorbed, calves are defined as having failure of passive transfer (FPT). Passive transfer of immunity is a critical factor and an important concern for the management of neonatal calf health. Despite the significant progress made in understanding biology and mechanisms of FPT, the application of this knowledge into improved treatments of sick calves has been limited because absorption of colostral components by the intestine of calves decreases quickly, and completely ceases approximately 24 h after birth, limiting the time available for the veterinarians to administer colostrum therapeutically (Berge et al., 2009). Immunoglobulin transfusion through plasma administration is empirically

recommended as a therapeutic intervention in calves with FPT (Weaver et al., 2000; Barrington and Parish, 2009) but the experimental data are rather controversial and there is no general agreement about the correct dosage of IgG in the treatment of FPT.

The specific objectives of this research are 1) to identify risk factors associated with outcome of the neonatal diarrhoea in calves undergoing standard therapy protocol. In particular to study the hematological, biochemical and clinical values to predict the fate of diarrhoeic calves, 2) compare sIgG and sTP in a control group of calves fed with a high quality colostrum vs. a treated group of colostrum–deprived calves submitted to an IV administration of IgG by plasma transfusion, in order to establish the dose of IgG which could provide a serum protective level (10 g/L).

1.2 Neonatal calf diarrhoea

Calf diarrhoea is attributed to both infectious and non-infectious factors. Multiple enteric pathogens (e.g., bacteria, viruses, and protozoa) are involved in the development of this disease. Co-infection is frequently observed in diarrhoeic calves although a single primary pathogen can be the cause in some cases. The prevalence of each of pathogen and disease incidence can vary by geographical location of the farms, farm management practices, and herd size (Foster and Smith, 2009). Currently, enterotoxigenic *Escherichia coli* (*E. coli*), rotavirus, coronavirus and *Cryptosporidium parvum* (*C. parvum*) appear to be the most significant infectious causes of calf diarrhoea (Foster and Smith, 2009).

1.2.1. Infectious etiologies

Escherichia coli

Escherichia coli can be classified into six pathogroups based on virulence characteristics: enterotoxigenic *E. coli* (ETEC), shiga toxin-producing *E. coli*,

enteropathogenic *E. coli*, enteroinvasive *E. coli*, enteroaggresive *E. coli*, and enterohaemorrhagic *E. coli* (Nataro and Kaper, 1998). Among these bacteria, the most common cause of neonatal diarrhoea is ETEC stains that produce the K99 (F5) adhesion antigen and heat-stable enterotoxin (STa) (Nataro and Kaper, 1998). Neonatal calves are most susceptible to ETEC infection during the first 4 days after birth and develop watery diarrhoea if infected (Foster and Smith, 2009). Following ingestion, ETEC infects the gut epithelium of the distal portion of small intestine and multiplies in enterocytes of the intestinal villi. Villous atrophy due to a loss of infected cells and damage to the laminar propria are commonly observed in affected small intestine (Foster and Smith, 2009). After colonization of the gut epithelium, heat-stable toxin production induced by ETEC leads to the up-regulation of chloride (Cl) secretion into the gut. This osmotically pulls water into the intestinal lumen and leads to the development of secretory diarrhoea in calves (Foster and Smith, 2009).

Rotavirus

Bovine rotavirus is a primary etiological agent of NCD. The virus belongs to the genus rotavirus within the family Reoviridae. Rotavirus is a non-enveloped virus possessing 11 double-stranded RNA segments and is very stable over a wide pH range with heat lability (Fenner et al., 2011). There are seven serogroups (A-G) of rotaviruses based on antigenic and genetic similarities of the intermediate capsid protein (VP6) (Steele et al., 2004). Group A rotaviruses are the major cause of rotaviral infection in domestic animals (Steele et al., 2004). Group A rotaviruses can be further classified into P or G types based on genetic and antigenic similarities of VP4 (protease sensitive protein) and VP7 (glycoprotein) which constitute the outer capsid of the virion and induce anti-viral neutralizing antibody production (Desselber et al., 2005). While VP4, VP6, and VP7 play a major role in maintaining viral structure, virus attachment, and antigenicity,

nonstructural glycoprotein 4 (NSP4) holds a special role as a viral enterotoxin. This protein also interferes with cellular homeostasis by elevating calcium (Ca) ion influx into the cytoplasm (Morris et al., 1999). These alterations account for drastic changes in the movement of nutrients and water across the intestinal epithelium and are more important for viral pathogenesis than histopathological lesions (Foster and Smith, 2009)

Bovine rotavirus usually causes diarrhoea in calves from 1 to 2 weeks of age. The milk uptaken by calves can provide a good environment for rotavirus survival under a wide range of gastrointestinal pH levels and infection of the intestinal epithelial cells (Dhana et al., 2009). Once infected, the calves shed a large amount of virus via feces for 5-7 days, thus contaminating the environment and allowing the virus to be transmitted to pen mates. The virus replicates in the cytoplasm of epithelial cells of small intestinal villi. Destruction of mature enterocytes in the villi, activation of the enteric nervous system by vasoactive components from the damaged cells, and secretion of a viral enterotoxin (e.g., NSP4) account for maldigestive/malabsorptive diarrhoea promoted by rotavirus infection.

Coronavirus

Bovine coronavirus is an enveloped virus with a single-stranded RNA genome (27~32 kb). Coronavirus typically affects calves within the first 3 weeks of life, and peak incidence occurs between the 7th and the 10th day. The virus is ingested from the environment, which is contaminated by other calves or adult cattles (Torres-Medina et al., 1985). The spike (S) protein of the virus plays an important role in virus entry and pathogenesis besides the ability to neutralize antibody (Lin et al., 2000). The S protein consists of two subunits (S1 and S2) and is crucial for virus-host interaction. While the S1 subunit facilitates binding of the virus to host cell receptors, the S2 subunit functions in the fusion of the

viral envelope to host cellular membranes (Yoo et al., 1991). Viral infection begins in the small intestine and usually spreads through the entire small intestine and colon. Microscopically, villi of the affected small intestine and colonic crypts become atrophic, and the lamina propria becomes necrotic. The virus replicates in enterocytes and progeny viruses are released through a normal secretory mechanism and cell lysis. Mature villous epithelial cells are the primary target of the virus although crypt enterocytes are also affected. Clinical signs in affected animals often have a longer duration due to the damage done to crypt enterocytes by the virus.

Cryptosporidium parvum

Cryptosporidium parvum is a protozoan parasite that is frequently associated with gastrointestinal tract disease in neonatal cattle. There are approximately 24 species of *Cryptosporidium* but *C. parvum* is considered the primary cause of calf diarrhoea and is a potential zoonotic agent (Fayer, 2010; Chalmers et al., 2011). Once *C. parvum* is ingested, the oocyst excitation releases sporozoites that penetrate enterocytes. The excysted parasites undergo asexual (type I meront) and sexual (type II meront) reproduction to produce macrogametocytes and microgametocytes. Upon fertilization of the macrogametocytes by microgametes, zygotes are developed with sporulates generating thin-walled oocysts involved in autoinfection. Next, thick-walled oocysts are eliminated by the host. The oocysts can survive for more than a month in the environment under favorable conditions (e.g., high temperature and moisture with low UV radiation) and are resistant to most disinfectants (Fayer et al., 1997). Environments contaminated with oocysts can be an immediate source of infection for calves. The invasion of *C. parvum* into enterocytes induces changes in intestinal cytoskeleton structures, such as loss of microvilli and shortening of columnar epithelial cells, leading to severe villous atrophy in infected animals

(Heine et al., 1984). Damage to the intestinal epithelium causes prolonged malnutrition and reduced growth rates in affected calves due to malabsorption and fermentation of undigested milk in the intestinal lumen (Nydam et al., 2005).

1.2.2 Clinical signs and clinicopathologic abnormalities in neonatal calves with diarrhoea

The systemic effects of diarrhoea are precipitated by two events: the loss of extracellular fluid and electrolyte, and the malabsorption of carbohydrates and their subsequent fermentation in the intestine (Argenzio, 1984; Lorenz, 2004a). Fecal water loss may be increased 28-fold, fecal volume 22-fold and fecal water content is increased from 73 to 94%. Renal water loss is reduced and there are severe losses of sodium (Na) and Cl ions and considerable losses of bicarbonate ions (HCO_3) (Lewis and Philips, 1978). Contraction of extracellular fluid volume gives rise to the clinical signs of sunken eyes and 'tenting' of skin folds; it leads to a fall in arterial blood pressure, which stimulates peripheral vasoconstriction. Peripheral vasoconstriction, in its turn, leads to poor tissue perfusion with a localized ischemia. The temperature of peripheral tissues falls approaching ambient temperature prior to death; the extremities, the ears and mouth, feel cold. Reduction of plasma volume leads to poor tissue perfusion, hypotension, lower metabolic activity, decrease of glomerular filtration and consequently, hyperkalemia and pre-renal azotemia (Trefz et al., 2013b). Metabolic acidosis is a frequently observed complication of neonatal diarrhoea in calves. Historically, metabolic acidosis and electrolyte imbalance in NCD were thought to be mainly associated with bicarbonate and electrolyte faecal loss. In presence of dehydration, reduced renal perfusion, and therefore reduced excretion of hydrogen ions and accumulation of L-lactate and other unidentified organic anions were considered to be the cause of this condition in the past (Kasari and Naylor, 1986; Kasari, 1999). Recently, scientific works have reported that D-lactate is a major component of high anion gap acidosis in NCD (Lorenz,

2004a). High D-lactate concentrations have been found in the feces of diarrhoeic calves (Ewaschuk et al., 2004). This finding suggested that the gut was the source of D-lactate and D-lactate was produced almost exclusively by microbes. The pathogenesis of D-lactic acidosis in diarrhoeic calves is hypothesized to involve decreased absorption of substrate with subsequent fermentation of this material in the gastrointestinal tract (Youanes and Herdt, 1987). The resultant drop in pH allows acid-resistant Gram-positive bacteria, particularly *Lactobacillus* spp., to proliferate and produce high concentrations of D- and L-lactate in the gut (Omole et al., 2001). Because D-lactate is metabolized by mammals at approximately one fifth the rate of L-lactate, it accumulates in the blood (Moller et al., 1997). Recent works reported that most clinical signs (alterations in posture, behavior and suckling reflex) of metabolic acidosis were attributed to an increase in blood levels of D-lactate (Lorenz, 2004b). D-lactate has a direct toxic effect on the brain. Abeysekara et al. (2007) suggested that D-lactate interferes with energy metabolism in the CNS by competitively blocking L-lactate entry into neurons, where L -lactate has an important role as a source of energy. Gentile et al. (2008) induced hyperchloremic metabolic acidosis with a mean base deficit of up to 22.4 mmol/L with an IV infusion of 4000 mL of a solution containing 400 mmol of hydrochloric acid in 0.9% NaCl over a period of 80 min in healthy calves. Despite the relatively severe acid-base imbalance during the entire observation period, no calves showed any clinical signs or depressed appetite. Abeysekara et al. (2007) have produced acidosis by infusion of either an iso-molar DL-lactic acid, L-lactic acid, or HCl, respectively, during a period of 6 h. Only DL-lactic acidosis was associated with severe disturbances in neurological functions. A further study of this subset of diarrhoeic calves with moderate to severe acidosis indicated that D-lactate, rather than metabolic acidosis *per se*, was associated with impaired posture, behaviour, and especially impairment of the palpebral reflex, whereas the sucking reflex appeared to be

influenced by dehydration and metabolic acidosis (Lorenz, 2004b). In an attempt to prove this hypothesis, D-lactataemia without acidosis was induced in a blinded study involving five clinically healthy calves by an injection of 100 mL of a 25% Na-D-lactate solution, while five control calves were given the same volume of 0.9% sodium chloride. The administration of D-lactate resulted in profound changes in posture and behaviour, while saline did not produce these changes (Lorenz et al., 2005). However, the injection of 100 mL of a 25% Na-L-lactate solution followed by infusion of 300 mL of the same solution over a period of 35 min did not trigger any clinical signs, so hypernatraemia and hyperosmolarity could be ruled out as reasons for the clinical signs in the study of calves with experimentally induced D-lactataemia (Gentile and Lorenz, 2005). These studies led to the conclusion that metabolic acidosis without appreciable increase in D-lactate concentrations had only minor influence on posture and behavior. However, it is still possible to determine bicarbonate requirements in calves with naturally acquired diarrhoea because of the significant correlation that exists between D-lactate concentrations and base excess values (Lorenz, 2004a). Trefz et al. (2012b) shown that the clinical parameters posture, behavior, and palpebral reflex were either closely correlated to base excess (BE) or D-lactate concentrations. These results demonstrated that the degree of metabolic acidosis in diarrhoeic calves can be predicted basing on clinical findings. Clinical signs provide a useful tool to determine bicarbonate requirements, but a revision is necessary for calves with ability to stand and marked metabolic acidosis (Trefz et al., 2012b).

In diarrhoeic calves, DL-hyperlactataemia induces an increase of anion gap (AG) (Ewaschuk et al., 2003). Anion gap was widely used in diagnosing metabolic acidosis in cattle and was used to differentiate metabolic acidosis caused by bicarbonate loss from the one caused by accumulation of organic acid (Constable et al., 1997; Stocker et al., 1999; Ewaschuk et al., 2003). Calculation

of AG is based on the principle of electroneutrality and is calculated as the difference between routinely measured cations and routinely measured anions (Constable et al., 1997). Values in normal animals have a pronounced variability and range between 7.1 to 22.6 mmol/L (Shull, 1978; Stocker et al., 1999; Omole et al., 2001; Ewaschuk et al., 2003). Normal positive value of AG representing the charge on serum proteins, phosphate, and strong anions, which are not routinely measured. Normally, approximately two thirds of the AG originates from the net negative charge of serum proteins (Constable et al., 1997). Increases in AG generally are indicative of a gain of organic anions such as ketoacids, DL-lactate or uremic anions. (Stocker et al., 1999; Constable et al., 1997; Ewaschuk et al., 2003). Decreases in AG generally are indicative of laboratory error, hypoalbuminemia, and reduction in sTP concentration in adult cattle (Constable et al., 1997; Kraut and Madias, 2007).

Hyperkalemia has been described as another clinically important electrolyte disturbance (Fisher and McEwan, 1967) that can result in skeletal muscle weakness and potentially life-threatening cardiac arrhythmias with concomitant progressive atrial standstill and prolonged ventricular depolarization (Lewis and Phillips, 1973; Trefz et al., 2013a). Hyperkalemia has traditionally been attributed to concomitant acidemia with intracellular buffering of hydrogen ions in exchange for potassium ions and impairment of the Na/K-ATPase (Lewis and Phillips, 1973; Constable, 2002). Recently Trefz et al. (2013b) have reported that dehydration is an important contributor to the pathogenesis of hyperkalemia and acidemia in neonatal calves with diarrhoea. Potassium filtered at the glomeruli is almost entirely reabsorbed by the proximal tubule cells, so that potassium excretion strictly depends on secretion by the distal tubules in exchange for Na and Cl ions (Sweeney, 1999). Experimental studies in rats have shown that this process is strongly influenced by urine flow and distal tubular delivery of Na (Good et al., 1984). Therefore, an increased proximal tubular reabsorption of

sodium and water in response to hypovolemia and a decrease in glomerular filtration rate due to hypovolemia result in reduced distal tubular potassium secretion and thereby an increased risk of hyperkalemia in calves with pre-renal azotemia (Trefz et al., 2013b).

Hypoglycemia frequently occurs in severe diarrhoea of calves, especially young calves near death. Anorexia, decreased absorption of nutrients, minimal glycogen reserves, inhibited gluconeogenesis, increased glycolysis due to reduced tissue perfusion and anoxia may contribute to hypoglycemia. Signs of hypoglycemia are weakness, lethargy, convulsions and coma.

1.2.3 Risk factors for calf diarrhoea

Several factors are associated with NCD hazard. Calf management, especially calving management (Lorino et al., 2005), colostrum management (Parè et al., 1993; Berge et al., 2009), calf housing and feeding (Nonnecke et al., 2003; Lundborg et al., 2005), farm size (Klein-Jöbstl et al., 2014) as well as hygiene (Bendali et al., 1999), have an important effect on calf's performance and health.

Difficult calving causes stress to the newborn calf, whith a decreased resistance to pathogens due to a combination of reduced calf vigour and delayed ingestion of colostrum. In addition, newborn calves which require assistance during parturition can be weakened for long periods after birth, and thus become exposed to more fecal pathogens than calves which stand up shortly after birth (Lorino et al., 2005). Dystocia affects the ability of the calf to suckle colostrum, resulting in decreased sIgG levels; consequently calves that survive dystocia are two to four times more likely to become sick in the first 45 days of life (Stott et al., 1976).

Although the relationship between FPT and mortality has been observed in many studies (Donovan et al., 1998; Tyler et al., 1998), the influence of FPT in

the development of diarrhoea is not clear (Berge et al., 2005). Donovan et al. (1998) showed that sTP was not a significant risk factor for diarrhoea and Berge et al. (2005) showed that adeguate passive transfer had no specific influence on number of diarrhoea days. Differently, Parè et al. (1993) showed that haematocrit and total protein may have important prognostic value in estimating the risk of calf diarrhoea. Immoglubulin and sTP were not associated with the age at onset of diarrhoea but a high IgG and a high sTP concentration were associated with a decreased length of episode (Parè et al., 1993). Moreover calves with FPT had 22% increased risk for more days with diarrhoea compared with calves with adeguate passive transfer (Berge et al., 2009). However, evidence from this and numerous studies reaffirm the belief that calves with adequate transfer of passive immunity have significantly less mortality and morbidity and consequently will receive fewer antimicrobial treatments (Berge et al., 2005).

Silper et al. (2014) showed that milk fed at amounts greater than 10 to 12% of calf body weight (BW) has a positive influence on calf's performance, whereas deficiencies in nutrition may lead to depressed immunity in calves and increase morbidity (Nonnecke et al., 2003). Individual calf housing is commonly advised because it may lead to a decreased pathogen load (Barrington et al., 2002). Housing of calves outside the barn is thought to reduce the risk of disease, as indoor housing has disadvantages such as an increased pathogen load and risk of disease transmission (Marcè et al., 2010).

Klein-Jöbstl et al. (2014) showed that the variable related to diarrhoea in farms was farm size. The authors speculated that the association between farm size and diarrhoea could be that although farms evaluated have become larger in recent years, additional personnel were not employed on the surveyed farms. Even in farms with up to 115 cows, no employees were present and this could lead to a decrease in the time that the farmer could spend on calf care. Furthermore,

Frank and Kaneene (1993) identified an increased incidence of calf diarrhoea for larger herds, explained by greater housing density that could lead to larger disease outbreaks. Kehoe et al. (2007) reported better colostrum management in smaller farms, which could also reduce the prevalence of disease in young calves. Cleaning calves area decreases the risk of diarrhoea (Bendali et al., 1999). When cows are dirty, their calves are logically more at risk of illness (Bendali et al., 1999). Removal of bedding is thought to eliminate a substantial portion of any fecal material harboring infectious organisms. A poorly drained area is likely to support the survival of pathogens and to increase the amount of contamination of the area. Pathogens can survive in the environment for months or years in cool, wet conditions, and consequently both the incidence and the mortality from diarrhoea increase with prolonged use of dirty calves area.

1.2.4 Prognosis of neonatal calf diarrhoea

Previous studies have reported the use of some clinical data, hematological and serum biochemical constituents for predicting the survival of diarrhoeic calves. Fayet and Overwater, (1978) have shown that the blood urea concentration had the best prognostic value and concluded that, by measuring two other parameters (the haematocrit and blood chloride concentration), they were able to classify the calves into two distinct groups (dead or survivor) with 80% accuracy. Klee et al. (1979) stated that treatment in diarrhoeic calves was less successful when hematocrit was over 50% and blood urea nitrogen concentration (BUN) was over 28.56 mmol/L. Seifi et al. (2006) showed that concentration of K was significantly higher in diarrhoeic calves that died than in diarrhoeic calves that survived. Calves with BUN levels above 13.07 mmol/L and K concentrations above 5.63 mEq/L were 5.6 and 4 times more likely to die, respectively (Seifi et al., 2006). Recent evidence suggests that calves with elevated D-lactate blood concentrations do not need additional specific therapy and D-lactataemia has no

impact on prognosis in calves with metabolic acidosis (Lorenz, 2004a; Lorenz and Vogt, 2006). Bacteremia is an important cause of morbidity and mortality in the large animal neonate and neonatal diarrhoea predisposes calves to septicemia (Lofstedt et al., 1999). Lofstedt and colleagues presented two models (a laboratory and a clinical model) for predicting septicemia based on all possible predictors studied. Fecteau et al. (1997) described a clinical score intended to be used on the farm and a more complete scoring system intended to be used in patients on which ancillary tests were performed. The study by Fecteau et al. (1997) indicated that the model used was a suitable tool for predicting bacteremia in ill calves in a clinical setting. Total clinical score (fecal score, hydration score, attitude score, umbilical score and scleral vessel score) was strongly associated with bacteremia (Fecteau et al., 1997). Risk of bacteremia increased with age, indicating that calves 1-week old or older were more at risk than younger calves. Lofstedt et al. (1999) showed that moderate (176–500 mmol/L) and marked (>500 mmol/L) increases in serum creatinine concentration, moderate and marked toxic changes in neutrophils, and FPT (IgG concentration $\leq$ 800 mg/dL and total serum protein $\leq$ 50 g/L) were associated with an increased risk of septicemia. In this study moderate and marked increases in serum creatinine concentration increased the risk of a calf being septicemic by 2-and 8-fold, respectively. The clinical model used by Lofstedt et al. (1999) showed that a significantly larger proportion of septicemic calves was <5 days of age. Moreover recumbency and absence of a suckling reflex were positively associated with an increased risk of septicemia (Lofstedt et al., 1999). Koch and Kaske, (2008) evaluated the clinical efficacy of IV administered hypertonic saline solution and hypertonic bicarbonate solution in the treatment of inappetent diarrhoeic calves. The results of this study have shown that treatment failed in 6 calves subjected to hypertonic saline solution and in 1 calf subjected to hypertonic bicarbonate solution. All treatment's failures had more

severe metabolic acidosis compared with successfully treated calves before treatment and no further differences between successfully treated calves and treatment failures were found (sTP, clinical data, glucose, potassium etc etc.).

1.3 Colostrum management for dairy calves

Ruminants have a synepitheliochorial placenta that separates maternal and fetal blood flows, allowing only small amounts of immunoglobulins to cross the placenta and reach the fetus (Peter, 2013). Consequently, at birth calves are hypogammaglobulinemic (Chigerwe et al., 2008a). They must acquire immunity passively through the consumption of colostrum, the first milk that a cow produces immediately after parturition (Kehoe and Heinrichs, 2007). Colostrum contains proteins such as immunoglobulins, lactoferrin, transferrin, albumin, α-lactalbumin and β-lactoglobulin; fat, carbohydrates, water, fat soluble vitamins; electrolyte, cytokines and growth factors such as lysozyme, lactoperoxidase, insulin, growth factor beta-2, growth hormone, insulin-like growth factor-I (Godden, 2008). Moreover colostrum contains immunologically active maternal leukocytes including macrophages, T and B lymphocytes and neutrophils (Le Jan, 1996). Colostrogenesis begins several weeks before calving, under the influence of lactogenic hormones and ceases abruptly at parturition (Foley and Otterby, 1978). Concentrations of colostrum components are higher in the first secretions harvested after calving (first milking colostrum), then decline steadily over the next six milkings (transition milk) to reach lower concentrations routinely measured in whole milk (Foley and Otterby, 1978).

After colostrum feeding, colostrum components are absorbed into neonatal circulation through intestinal epithelium and enhanced immunological response to specific and nonspecific mitogens (Godden, 2008), increase phagocytosis and bacterial killing ability, stimulate humoral immune responses (Le Jan, 1996),

increase intestinal mucosal growth, brush-border enzymes, intestinal DNA synthesis, villus size, glucose and amino acids uptake (Baumrucker et al., 1994; Bird et al., 1996; Buhler et al., 1998). If a calf does not receive an adequate amount of colostrum, or if the colostrum is of poor quality, the calf may suffer of FPT. Failure of passive transfer of immunity is defined as calf sIgG levels less than 10 g/L or serum total protein (sTP) less than 52 g/L (Tyler et al., 1996; Calloway et al., 2002). Failure of passive transfer predisposes calves to infection, increases risk of morbidity and mortality, leads to decreased growth rate and reduced lifetime milk production (Quigley et al., 1995; Rea et al., 1996; Quigley et al., 1997; Donovan et al., 1998; Weaver et al., 2000; Chigerwe et al., 2009).

1.3.1 Factors influencing failure of passive transfer of immunity
Absorption of immunoglobulins
Cells in the immature small intestine absorb macromolecules and leukocytes via pinocytosis in a non-selectively manner. The colostrum components move through the epithelium, into the lymphatic system and then in the bloodstream (Quigley et al., 2005). Gut cell maturation and other changes such as a decrease in the pH of the abomasum and the initiation of intestinal digestive enzyme secretion prevent absorption of intact IgG and leukocytes starting at birth, with complete cessation of non-selective absorption occurring by 24 hours of life (Quigley et al., 2005).

Timing of colostrum intake
Beam et al. (2009) have reported that calves fed with colostrum more than 4 hours after birth were 2.7 times more likely to be susceptible to FPT than calves fed with the first colostrum within 4 hours. As optimal absorption occurs within 4 hours of birth and declines rapidly after 6 hours, calves should ideally be fed

within the first 4 hours of birth, with the first feeding not later than 6 hours after calving (Davis and Drackley, 1998; Godden, 2008).

Volume of colostrum fed

Davis and Drackley, (1998) showed that the minimum amount of IgG that should be consumed was 100 grams, though some literature suggests that 150 to 200 grams is more appropriate (Chigerwe et al., 2008b). Calves fed with more than 100 g of IgG in the first feeding had a low prevalence of FPT (Besser et al., 1993). Several studies have reported that calves fed with a larger volume of colostrum (4 L vs. 2 L in the study of Morin et al., 1997, and 3 L vs. 1.5 L in the study of Godden et al., 2009a) had significantly higher sTP levels. Trotz-Williams et al. (2008) showed that the risk of FPT decreased as the volume of colostrum fed in the first 6 hours of life increased. Calves that ingest ≥3 L at the first feeding will need to ingest 1 L of colostrum within 12 hours to have optimum colostral intake (Chigerwe et al., 2009). Calves that fail to ingest at least 1 L within 12 hours should be tube fed with 2 L of colostrum. Calves that ingest >2 L but <3 L of colostrum 1, 2, 3, or 4 hours after birth will require a minimum intake of an additional 2 L of colostrum within 12 hours of age. Calves that do not ingest at least 2 L within 12 hours should be tube fed with 2 L of colostrum at that time. Calves that ingest <2 L at their first feeding should be targeted for immediate oroesophageal tube feeding, and a total volume of 3 L should be administered in the first feeding. Calves in this category probably will need to ingest ≥1 L of colostrum at the second feeding to have optimum colostral intake. If they ingest <1 L, they should be tube fed with 2 L of colostrum (Chigerwe et al., 2009). The authors suggested that these recommendations are sufficient to maintain FPT rates <10% (Chigerwe et al., 2009).

Quality of colostrum fed

Although it is recognized that colostrum contains a wide spectrum of important immune and nutritional components, because of the relationship between Ig concentrations and calf health is understood better, and because IgG composes more than 85% of total Ig in colostrum, the concentration of IgG in colostrum has traditionally been considered the hallmark for evaluating colostrum quality (Godden, 2008). High-quality colostrum has an IgG concentration greater than 50 g/L (McGuirk and Collins, 2004).

The volume of colostrum produced is one factor that affects the concentration of IgG. Morin et al. (2010) reported that for every L of colostrum produced, IgG concentration decreases of 3.7%, suggesting that colostrum should be harvested as soon as possible after calving. The transport of IgG into the mammary glands stops abruptly at calving although milk production continues. This results in a dilution of IgG concentration as a consequence of increased time of colostrum harvesting. A study by Holloway et al. (2001) indicated that both fresh and frozen colostrum could provide adequate amounts of IgG. In that study, 26 calves were fed either with fresh or frozen-thawed colostrum from the same pool, and serum IgG was determined at 48 hours. There was no significant difference in either serum or colostral IgG between the treatment groups. In another study, no difference was found in IgG content among fresh, frozen, or refrigerated colostrum, however refrigerated colostrum had significantly higher total coliform plate count (TPC) compared with fresh or frozen samples (Morrill et al., 2012). Colostrum containing >50 g/L of IgG, and containing < 100,000 cfu/mL of bacteria is considered a good quality colostrum and should be fed to calves (Morrill et al., 2012). Bacterial contamination of colostrum is a concern because free IgG in the colostrum can be bound to bacteria in the gut thus preventing absorption, or the same bacteria can block absorption by binding directly to enterocytes (Godden, 2008). As the gut non-selectively absorbs

molecules, it will also absorb harmful molecules such as pathogenic bacteria. It is therefore crucial to ensure that the calf receives as much clean, good quality colostrum as soon as possible after birth so that IgG, rather than pathogens, is absorbed and enters in the bloodstream (Quigley et al., 2005). Bacterial contamination can be avoided through hygienic preparation of the dam's teats and maintaining a clean and well-functioning milking and storage equipment (McGuirk and Collins, 2004). Colostrum stored in a refrigerator has higher coliform counts compared with fresh or frozen colostrum, however refrigerated colostrum has the lowest somatic cell count (SCC) compared with fresh or frozen colostrum (Morrill et al, 2012). Freezing high quality colostrum is an appropriate storage method (Quigley et al., 2005). Pooling colostrum from several cows is a practice used in some dairy farms. A review by Weaver et al. (2000) showed that pooling colostrum decreased the concentration of IgG. Pooling can also increase disease exposure as calves are receiving colostrum from many different cows (McGuirk and Collins 2004). Beam et al. (2009) indicated that approximately 20% of dairy farms in the USA used pooled colostrum. They found that farms that pooled colostrum were 2.2 times more likely to have calves with FPT compared with those that did not pool. Samples of colostrum from individual dams had significantly greater IgG concentrations and lower SCC and TPC compared to pooled samples in a survey of American farms (Morrill et al., 2012).

One management practice demonstrated as a way to reduce bacterial contamination of colostrum is pasteurization; however, there is evidence that suggests that certain methods of pasteurization can destroy Ig. Godden et al. (2003) looked at the effects of batch pasteurization at 63°C and found that colostrum pasteurized in large batches (95 L) had to be heated for 2.5 to 3 hours in order to reach a temperature of 63°C, and had a 58.5% reduction in IgG. Colostrum pasteurized at 63°C for 1 hour in small batches (57 L) had a 23.6%

reduction in IgG. Pasteurizing colostrum for 60 minutes at 60°C was shown to be very effective in reducing total bacterial count while preserving IgG levels (Godden et al., 2006). This indicates that it is possible to effectively pasteurize colostrum, but care must be taken to ensure that the appropriate method is used to preserve IgG while reducing the pathogen load.

Factors unrelated to colostrum that affect failure of passive transfer of immunity

Donovan et al. (1986) reported that dystotic calves had a significantly lower sTP compared to calves with an easy birth. Similarly, another study found the main causes of FPT were low volume of colostrum intake and poor vigor associated with dystocia (Furman-Fratczak et al., 2011). This same study described that calves from primiparous cows were more at risk of FPT than those from multiparous cows. Decreased colostral Ig absorption in the first 12 hours has been reported in calves with postnatal respiratory acidosis, associated with prolonged parturition (Besser et al., 1990). Although hypoxic calves may have delayed IgG absorption initially, studies have reported that there is no difference in overall absorptive capacity between hypoxic and normoxic calves and that there is no difference in serum IgG concentrations by the time of gut closure (Tyler and Ramsey, 1991). Weaver and colleagues (2000) suggested that an increased rate of FPT seen in calves with metabolic or respiratory acidosis may be caused by a delay in the animal getting up to nurse, not by reduced absorptive capacity.

1.3.2 Economic importance of colostrum ingestion in dairy calves

The prevalence of FPT in dairy calves in the US ranged between 19 to 40% (USDA-APHIS, 1993; Tyler et al., 1998; Beam et al., 2009). The prevalence of FPT in dairy calves in Canada was 37% and in Australia 38% (Trotz-Williams et al., 2008; Vogels et al., 2013). To the author's knowledge, there are not published

data on the prevalence of FPT in Europe. Failure of passive transfer is responsible for approximately 50% of calf death losses in US dairy farms (Tyler et al., 1999a). Calves with failure of passive transfer have an increased mortality due to *Escherichia coli* septicemia (Sawyer et al., 1973). Inadequate ingestion of colostral immunoglobulins affects calf's health and survival beyond the neonatal period; calves with FPT have an increased rate of mortality until at least 10 weeks of age (Tyler et al., 1998). In another study, calves with FPT had 2-times higher odds of pneumonia than calves with adequate passive transfer during the first 3 months of life (Virtala, 1999). Higher serum IgG concentrations (> 12 g/L) from 24 to 48 hours of age in heifer calves are associated with increased daily weight gain up to 180 days of life and higher weaning weights compared to calves with low serum IgG concentration (Robinson et al., 1988). Calves with FPT have lower milk production in their first lactation and are more likely to be culled for low production (DeNise et al., 1989).

1.3.3 Measures of failure of passive transfer of immunity

Several tests are available to assess passive transfer colostral immunoglobulin in dairy calves. The most commonly used laboratory tests include radial immunodiffusion (RID), enzyme-linked immunosorbent assay (ELISA) and the turbidimetric immunoassay (TIA). Although RID has long been considered the "gold standard for testing" (Fahey and McKelvey, 1965) there are several problems with RID and it may not be optimal for all situations (e.g., colostrum testing) (Quigley, 2008). Another disadvantage of the method is the prolonged time (48-72 hours) before results are available. The TIA test is a procedure similar to the RID, but in a liquid medium rather than using agar. This gives the TIA technique the advantage of being much faster and, moreover, it can be automated. Rather than reading ring diameters (as is done with RID), the turbidity (cloudiness) of the solution can be measured with a machine, which can

dramatically increase the number of samples that can be done in a day. ELISA techniques are more flexible and complex. It has many advantages compared to other methods, including speed, accuracy and being able to measure very small quantities of IgG. It is a more complex method and requires greater technical skill and equipment compared to the other techniques. Therefore, it tends to be less widely used in measuring total IgG concentration in calf serum studies (Quigley, 2008). Any of this laboratory methods can be very accurate and precise (Quigley, 2008) but they all are too complex and expensive for routine use on farm. Thus, a rapid, accurate, and inexpensive method to estimate IgG concentration in serum is essential for identification calves with FPT.

The most common rapid calf-side test to determine serum IgG concentration includes sTP by refractometry, sIgG concentration by BRIX refractometry and immunoassay kit (Dawes et al., 2002). Serum total protein measurement with refractometry measures total globulins and other proteins. A total serum concentration of 52 g/L is equivalent to 10 g/L of sIgG (Tyler et al., 1996). This endpoint is used to indicate adequacy of passive transfer in clinically normal calves IgG (Tyler et al., 1996). In clinically ill animals a serum total protein endpoint of ≥55 g/L is used to indicate adequate passive transfer to account for dehydration (Tyler et al., 1999b). Serum total protein refractometry is the test recommended for herd monitoring of calves for adequate transfer of colostral immunoglobulins (Weaver et al., 2000). Most studies establishing cutoff values of TP or IgG for determination of FPT have been based on data primarily obtained from Holstein calves. Villarroel et al. (2013) showed that the cutoff values established for sTP or sIgG in Holstein calves are not appropriate to be used in Jersey calves. This data suggests that there are significant breed differences, demonstrating the need for future research to established more accurate breed reference values. In addition, sTP concentration varies considerably due to the type of substrate used. Cut-off point for FPT has been

associated with a serum total protein concentration of 52 g/L (Tyler et al., 1996). Using the regression equation derived in the study by MacFarlane, et al. (2014) a serum protein concentration of 52 g/L equated to a plasma total protein measurement of 56 g/L. Brix refractometer can be used to evaluate IgG concentrations in serum (Morril et al., 2013). The cut point of 7.8% Brix has a high percentage of correctly classified samples (90.8%) and may be used as the cut point to identify failure of passive transfer in 1-d-old calves (Morril et al., 2013).

An ELISA which directly measures serum IgG concentration is available for assessing passive transfer (Dawes et al., 2002). The sensitivity and specificity of the immunoassay in detecting calves with inadequate passive transfer of colostral immunoglobulins is 0.93 and 0.88 respectively. The ELISA can be used in farms and does not require additional instrumentation needed for refractometry and sodium sulfate tests.

1.3.4 Management of failure of passive transfer of colostral immunoglobulins in dairy calves

Calves with FPT will survive if housing, nutrition and hygiene are optimal (Weaver et al., 2000). The decision to treat calves with FPT should be based on calf's age, value and presence of other risk factors for morbidity and mortality in calves (Weaver et al., 2000). Prophylactic antibiotics have been suggested as an adjunct treatment in calves with failure of passive transfer (Weaver et al., 2000). Management of FPT in clinically normal calves involves transfusion with plasma, serum or whole blood intravenously or intraperitoneally (Weaver et al., 2000). However treatment is empirical and no controlled studies have evaluated the efficacy of plasma, serum or whole blood on morbidity and mortality in calves with FPT. Gamma immunoglobulin transfusion through plasma administration has been recommended empirically as a prophylactic intervention in calves with

inadequate transfer of colostral immunity (Smith and Little, 1922; Anderson et al., 1987; Selim et al., 1995; Quigley and Welborn, 1996; Weaver et al., 2000; Barrington and Parish, 2009; Chigerwe and Tyler, 2010). The experimental data are rather controversial, and there is no general agreement about the correct dose of IgG in the treatment of FPT. A considerable amount of literature evaluated failed to specify the effectiveness of transfused IgG to reach serum protective levels (>10 g/L) due to a lack of data concerning the sIgG concentration pre and post plasma transfusion, due to a lack of concentration of IgG in the transfused products or due to injection of a small dose of IgG.

Chapter two

Identifying factors associated with mortality of dairy calves with neonatal diarrhoea: A retrospective study (2006 to 2014).

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Abstract

The importance of neonatal calf diarrhoea has prompted the review of clinical and laboratory data in diarrhoeic calves up to 30 days old presented to a referral institution to identify the major risk factors associated with outcome in calves undergoing standard therapy protocol.

Clinical signs, complementary examination findings and outcome were reviewed in 131 calves diagnosed with neonatal diarrhoea at the Clinics of Ruminant, Swine and Management, University of Milan between 2006 and 2014.

After therapy 86 (66%) calves were discharged in a healthy state, 45 (34%) calves died. Logistic regression analysis showed that serum total protein concentration, anion gap value and hospitalization in cold months were the major risk factors associated with mortality in diarrhoeic calves, whereas a great importance was found in serum total protein concentration.

These results suggested that serum total protein concentration could assist the veterinarian in distinguishing between diarrhoeic calves with positive prognosis and diarrhoeic calves with major risk of treatment failure.

Keywords: Calves, Neonatal Diarrhoea, Failure of Passive Transfer, Colostrum, Outcome.

Introduction

Neonatal calf diarrhoea is a serious welfare problem and a cause of economic loss due to mortality, treatment costs and poor growth (Svensson et al., 2006; Østerås et al., 2007; USDA-APHIS, 2007; Torsein et al., 2011). Calf diarrhoea is an example of a complex and multifactorial disease, resulting as it does from an interaction among the calf, its environment, nutrition and infectious agents (Bendali et al., 1999; Weaver et al., 2000; Nonnecke et al., 2003; Berge et al., 2005; Berge et al., 2009; Pithua et al., 2009). *Cryptosporidium* spp., bovine rota and corona viruses and *Escherichia coli* F5 are the most important pathogens causing neonatal diarrhoea in calves worldwide (Foster and Smith, 2009). Although different studies have aimed to identify risk factors for the presence of calf diarrhoea in the farms (Parè et al., 1993; Bendali et al., 1999; Berge et al.,2009; Pithua et al., 2009; Klein-Jöbstl et al., 2014), little is known about factors involved in the outcome in affected calves. Fayet and Overwater (1978) reported that the blood urea nitrogen (BUN) concentration had the best prognostic value in calves with neonatal diarrhoea and concluded that by adding two other easily measured hematological parameters (the haematocrit and the blood chloride concentration) the probability of classifying correctly the calves into one or another group (dead or surviving) was approximately 80%. Klee et al. (1979) stated that treatment in diarrhoeic calves was less successful when haematocrit was over 50% and BUN was over 28.56 mmol/L. Recent evidence suggests that calves with elevated D-lactate blood concentrations do not need additional specific therapy and D-lactataemia has no impact on prognosis in calves with metabolic acidosis (Lorenz, 2004a; Lorenz and Vogt, 2006). Seifi et al. (2006) showed that concentration of potassium (K) was significantly higher in diarrhoeic calves that died than in diarrhoeic calves that survived. Hematocrit, BUN, creatinine and K may have animportant prognostic value in figuring out the outcome of calf diarrhoea. The results of this study showed that diarrhoeic

calves with BUN levels above 13.07 mmol/L and K concentrations above 5.63 mEq/l were 5.6 and 4 times more likely to die, respectively (Seifi et al., 2006). Koch and Kaske, (2008) evaluated the clinical efficacy of IV administered hypertonic saline solution and hypertonic bicarbonate solution in the treatment of inappetent diarrhoeic calves. In this study, treatment failed in 6 calves subjected to hypertonic saline solution and in 1 calf subjected to hypertonic bicarbonate solution. All treatment failures had more severe metabolic acidosis compared with successfully treated calves before treatment and no further differences between successfully treated calves and treatment failures were found. Unnecessary treatment or treatment of every ill calf may result in needless expense, treatment side effects, drug residues, and increased resistance to antimicrobials (Fecteau et al., 1997). A model capable of predicting mortality in diarrhoeic neonatal calves could be helpful for the veterinarian in distinguishing between diarrhoeic calves with positive outcome and calves with high mortality risk. The objective of this retrospective study was to review clinical data and ancillary test examination findings on outcomes in dairy calves diagnosed with NCD referred to the Clinics of Ruminant, Swine and Management (CRSM), University of Milan (Italy), in order to identify major risk factors associated with outcome of the neonatal diarrhoea in calves undergoing standard therapy protocol.

Materials and methods

Animals

Medical records of all dairy calves admitted to the CRSM between January 2006 and July 2014, up to 30 days of life, with a clinical diagnosis of NCD were reviewed. For each case, initial data extracted from the medical record included age, sex, breed, weight, month of hospitalization, clinical presentation at

hospitalization, therapy and outcome (live calf or died calf). The management of all calves undergoing the study was within standard protocols of the clinic for the treatment of NCD, therefore no animal ethics approval had to be sought. Exclusion criteria included the presence of obvious clinical signs of concurrent diseases such as pneumonia, fractures, congenital condition, surgical abdomen, septic arthritis, severe ruminal drinking or umbilical disorder. Furthermore, calves without venous blood gas analysis, or sTP concentration, clinical data or calves undergoing different therapy protocols, were excluded from the study. Because of regional preferences, most of dairy calves (90%) were Holstein Friesians, only this calves were admitted to the study.

Clinical and laboratory examination

Clinical diagnosis of neonatal diarrhoea was based on specific clinical signs: watery feces, dehydration, reduction of suckling reflex and dullness. All physical examinations followed a standardized protocol and were carried out at the time of admission by one of the main authors. The degree of dullness was evaluated using a 5-point vitality system (Table 1). The hydration status of each calf was evaluated using a 4-point dehydration scoring system assessed by estimating the degree of enophthalmos, the duration of skin tenting on the upper eyelid and the behavior of the calves (Table 2). After admission, calves were monitored by a daily clinical examination. The results of the following ancillary procedures, on the day of admittance to the CRSM, were recorded: venous blood gases analysis and serum total protein (sTP) concentration. Blood samples were collected from jugular vein into Vacutainer™ tubes with no additive for evaluation of sTP concentrations and allowed to clot and then centrifuged at 20°C for 10 minutes at 900 X g. Serum TP was estimated using standard quantitative colorimetric assay biuret method for serum and plasma (total proteins quantitative colorimetric assay biuret method on serum and plasma; Biochemical Enterprise,

Milano Italy), according to manufacturer's protocol. The colorimetric results were determined by a clinical chemistry analyzer (Roche Cobas Mira Classic; Hoffmann–La Roche, Basel Switzerland). Blood samples for gas analysis were anaerobically collected from jugular venipucture by disposable 2.5 mL syringe which contained anticoagulant (0.1 mL sodium heparin) and blood pH, bicarbonate, partial pressure of carbon dioxide (pCO_2), sodium (Na), chloride (Cl) and K concentrations were immediately determined using a blood pH, gas-analyzer (AVL Opti CCA Diamond Diagnostic, Holliston, MA, USA).

Treatment

After blood samples therapy was performed according to standard protocols. Diarrhoeic calves that were neither dehydrated nor acidemic, with a good suckling reflex, received on admission 1 L of an oral rehydration solution (ORS). Calves that did not drink ORS completely, received the remaining after two hours. One liter of a standardized oral rehydration solution was additionally offered three times within the 24 hours period between milk feedings. Treatment of acidemic calves was performed by intravenous hypertonic solution (Lorenz and Vogt, 2006) after introducing a venous catheter (Introcan Safety 22G x 25 mm, BBraun, Melsungen, Germany) into the auricular vein. Calves with metabolic acidosis and grade 1 of dehydration (no enophthalmos) received a constant drip infusion (rate 20 to 30 mL/kg/h) consisting of 5 L bags of isotonic saline spiked with an amount of 8,4% sodium bicarbonate (Grove-White, 1996; Lorenz and Vogt, 2006; Berchtold, 2009). After the hypertonic solution, calves with acidosis and dehydration degree ≥10% received an additional 5 L bags of isotonic saline solution at slow infusion rate (10-15 mL/kg/h). The provided amount of sodium bicarbonate was adapted for each individual calf and calculated as follows (Lorenz and Vogt, 2006):

$$\text{bicarbonate requirements (g)} = \text{body weight (kg)} \times \text{BE (mmol/L)} \times 0.6 \text{ (L/kg)} \times 0.084 \text{ (g/mmol)}$$

Calves with a history of malnutrition or lack of milk intake for more than 12 hours received 200 mL of a 50% glucose solution added to the infusion solutions. At the day of hospitalization all calves received flunixin meglumine (Alivios, Fatro S.p.a., Ozzano dell'Emilia, BO, Italia) at a dose of 2.2 mg/kg intravenously. Amoxicillin-clavulanic acid (Synulox, Zoetis Italia s.r.l., Roma, Italia) was administered subcutaneously (SC) at a dose of 10 mg/Kg for 5 days. Furthermore all calves received a preparation with vitamin E and selenium SC (Fatro S.p.a., Ozzano dell'Emilia, BO, Italia) (0.3 mg/kg sodium selenite and 10 mg/kg vitamin E).

Feeding and housing
After hospitalization calves were placed in individual hutches (1,8×1,2 m) with a straw litter and fed with milk replacer. Powdered milk sostitute (120 g/L) corresponding to 10% of BW per day, was offered via nipple buckets divided into 3 meals (8:00 AM, 1:00 PM, 6:00 PM). Calves without signs of dehydration and not acidemic received ORS contained 4 g NaCl, 20 g glucose, 3 g KHCO3, and 3 g sodium propionate per liter. Fresh water, hay and calf starter were offered ad libitum.

Assessment of therapeutic success and failure
Therapy was considered to be successful if vitality score, hydration score, suckling reflex and milk intake improved. A treatment failure was defined as a deterioration (or failed improvement) of the general condition 24h after hospitalization or deterioration after a primary improvement. In these cases further IV fluid treatment based on the actual acid–base status, clinical dehydration and the assumed on-going losses of fluids and buffer substances

was performed. Calves were discharged in a healthy state as defined by a normal hydration score, high vitality score, normal suckling reflex, good appetite and normal consistency of the feces for at least two days. The outcome of the disease was coded as 0 or 1 if the calf survived or died, respectively.

Data analysis

A logistic regression was used to estimate the probability of the disease outcome (0/1) based on the available medical records. The sex of the calf (male/female) and the month of hospitalization were also included as fixed effects. In order to built the predictive model a cross validation procedure was adopted (Hastie et al., 2009). Firstly the 131 was randomly split in a *training* and a *testing* datasets which included 75% (n=92) and 25% (n=39) of the observations, respectively. Then, the 92 samples from the *training* dataset were used to build the predictive model with a 5-fold cross validation scheme. The 92 samples were randomly split into 5 subsets of approximately the same size. In turn, the observations in one subset were set to missing and predicted using the model trained with the remaining four subsets, until all subsets were used once as validation set. This process was repeated 10 times, each time randomly sampling different subsets, eventually yielding 50 replicates of the analysis. Finally, the obtained predictive model was used with the *testing* data to predict the probability of the disease outcome (dead/survived). Before implementation of the cross-validation procedure data were checked for multicollinearity. Indipendent variables were also standardized before entering the model. For the *training* and the *testing* dataset the following statistics were obtained: 1) the Accuracy (AC), i.e. the proportion of the total number of predictions that were correct, 2) the Sensitivity or true positive rate (TPR), i.e. the ability of the test to identify a condition correctly, 3) the Specificity or True Negative Rate (SPC), i.e. the ability of the test to exclude a condition correctly. The absolute value of the t-statistic for each

model parameter included in the predictive model was used for estimating the contribution of each variable to the model itself. Data preparation, analysis and figures were produced with the open source statistical environment R[1], sing the packages *plyr* (Wickham, 2011)*, ggplot2* and *caret* (Kuhn, 2008).

Results

A dataset of 384 calves was reviewed. Based on our selection criteria, medical records from 131 holstein calves were included in this study. There were 115 females and 16 males. The average body weight was 42.3 kg. All medical records of the admitted calves are summarized in Table 3. The 131 calves were assigned to treatment standard protocols based on clinical and acid-base status: oral rehydration solution (n=16), calves with metabolic acidosis and grade 1 of dehydration (n=39), calves with metabolic acidosis and grade 2/3 of dehydration (n=76). Glucose was added to infusion fluids in 108 cases. After therapy 86 (66%) calves were discharged in a healthy state, 45 (34%) calves died. Calves discharged in a healthy state and treatment failure for each month of the study are presented in Table 4. Correlation between age (days), venous blood gases analysis, sTP, dehydration score, vitality score and rectal temperature are summarized in Figure 1. The strongest correlation were observed between HCO3 and BE (r=0.97) and HT and Hb (r=0.93). Ht and BE predictors were not included in the final model. Accuracy, sensitivity and specificity estimated from cross-validation procedure for *Training* and *Testing* data are in Table 5. The contribution of each predictors to the final predictive model can be observed in Figure 2. Logistic regression analysis shows that sTP concentration, AG and hospitalization in cold months are the major risk factors associated with mortality in diarrhoeic calves, whereas a great importance was found in sTP concentration. The relationship between survived/deceased calves and their sTP concentration are shown in Figure 3.

Discussion

Neonatal calf diarrhoea is the most common cause of morbidity and mortality in preweaned dairy calves worldwide. In calves aged <31 days, NCD is the most common cause of death with a peak probability of dying due to enteritis during the second week of life (Svensson et al., 2006; Windeyer et al., 2014). The objective of this study was to evaluate the major factors associated with mortality in dairy calves affected by neonatal diarrhoea.

Results showed that 34% of calves died during hospitalization. Previous studies have reported that mortality risk for diarrhoea ranged from 4.9% to 24% in field conditions (Fecteau et al., 1997; Windeyer et al., 2014), and 5% to 35% in hospital condition (Lofstedt et al., 1999; Seifi et al., 2006; Trefz et al., 2012a). In our study, mortality rate was congruent with that of the models for prediction of septicemia by Lofstedt et al. (1999). Results of this study have shown that 81% of nonsepticemic calves were discharged from the hospital compared with only 29.5% of septic calves. Clearly, studies conducted on naturally infected calves in a hospital setting create a substantial increase of mortality rate. This is not in agreement with previous literature that suggests that mortality rate, in hospital setting, was only 5% (Trefz et al., 2012a) Discrepancy may be due to the greater severity of clinical signs of our calves than Simmental calves of the study by Trefz and colleagues. In this study at the time of admission most calves were able to stand up after encouragement and the majority of the calves did not show or had a lower degree of enophthalmos. On admission, our calves showed a lower vitality score (mean value 2.61±1.08) and a higher dehydration score (mean value 1.80±0.74). Clinics of Ruminant, Swine and Management of University of Milan was founded in 2005 and is a much more younger clinic than other important European and North American clinics for ruminants. Our point of view is that it is very important to increase awareness of farmers about clinicopathologic changes in neonatal calves with diarrhoea. Indeed, early

recognition and treatment of diarrhoea are essential to improve survival and to decrease the occurrence of a sequela of a bacteremic episode. In recent years, with this awareness work we noticed a great demand of veterinary assistance with further improvement of clinical signs at hospitalization and consequently, increased survival rate compared to previous years of clinical activity. Another possible explanation for this results is that calves used in the study of Trefz et al. (2012a) were Simmental breed. It is reasonable to assume that there are significant breed differences, e.g., the differences in colostrum properties could potentially alter immunoglobulin absorption and create significant differences in passive immunity serum that alter morbidity and mortality rate between the two breeds (Godden, 2008; Villarroel et al., 2013).

Studying the factors associated with mortality helped gain a better understanding of risk factors associated with disease, improving prognosis and treatment. In the past two decades an increasing interest toward NCD hazard has been used to improve calf management practices (Parè et al., 1993; Bendali et al., 1999; Berge et al.,2009; Pithua et al., 2009; Klein-Jöbstl et al., 2014). However, a few observational studies were conducted to determine the pathologic factors associated with outcome in naturally infected calves. Some studies directly evaluated haematological and serum biochemical constituents to predict the survival of diarrhoeic calves (Fayet and Overwater, 1978; Klee et al., 1979; Seifi et al., 2006), others evaluated the impact on therapy of a single pathologic substrate (Lorenz, 2004; Lorenz and Vogt, 2006). Mortality rate (and consequently related data), can be extrapolated from other research models but the evidence regarding this data is often not reported. Diarrhoeic calves are widely used in prospective and controlled trials to evaluate the effectiveness of therapeutic protocols or to develope a diagnostic method (Koch and Kaske, 2008; Sen et al., 2009; Bellino et al., 2012; Trefz et al., 2012b) but it is difficult to

compare the prognosis data with others in the literature because few reports include a detailed outcome data.

In our study, only sTP concentration, AG and cold season increased the odds of dying in diarrhoeic calves.

Mean sTP value obtained in this study is consistent with a poor passive immunity (<55g/L). Furthermore, our results have shown that sTP represents a major factor associated with mortality of dairy calves with neonatal diarrhoea. It is the first study, to the author's knowledge, that shows this result. When adequate amounts of immunoglobulins are not absorbed, calves are defined FPT if either sIgG or sTP concentration are less than 10 g/L and 52 g/L, respectively (Tyler et al., 1996). In clinically ill animals a serum total protein endpoint of ≥55 g/L was used to indicate adequate passive transfer (Tyler et al., 1999b). Although the relationship between FPT and mortality has been observed in many studies (Donovan et al., 1998; Tyler et al., 1998; Tyler et al., 1999a; Virtala et al., 1999), the influence of FPT on the development of diarrhoea was not clear or was undebatable (Berge et al., 2005; Koch and Kaske; 2008; Berge et al., 2009; Chand et al., 2009; Furman-Fratczak et al., 2011). Some studies have shown no significant effect of colostrum feeding routines on the risk of diarrhoea or on the risk of shedding *C. parvum* (Trotz-Williams et al., 2007; Gulliksen et al., 2009). This lack of a significant effect may be explained by a high number of diarrhoea cases caused by *C. parvum*, for which colostral IgG is less protective (Trotz-Williams et al., 2007). Donovan et al. (1998) showed that sTP was not a significant risk factor for diarrhoea and Berge et al. (2005) showed that adequate passive transfer had no specific influence on number of diarrhoea days. Contrary to these results, Parè et al. (1993) showed that sTP could have important prognostic value in estimating the risk of calf diarrhoea. Immoglubulin and sTP were not associated with the age at onset of diarrhoea but a high IgG and a high sTP concentration were associated with a decreased length of diarrhoea episode.

Moreover calves with FPT had 22% increased risk for more days with diarrhoea compared with calves with adequate passive transfer (Berge et al., 2009). As shown in Fig. 3, deceased calves were distributed over the low range of sTP concentration. Colostrum contains IgG, active leukocytes and other important substances that are involved in natural host defense system against local invading microorganisms (Godden, 2008). In diarrhoeic calves FPT is an important predisposing cause for neonatal septicaemia (Fecteau et al., 1997; Lofstedt et al., 1999). Sepsis is an important cause of mortality in the large animal neonate and neonatal diarrhoea predisposes calves to septicemia (Lofstedt et al., 1999). On the basis of this observation, it could be speculated that passive immunity (i.e. sTP) is the most significant factor that contributes to a decreased efficiency of therapeutic protocols and has a great impact on outcome of NCD.

Our results showed that AG was associated with outcome of dairy calves with neonatal diarrhoea. Anion gap was widely used in diagnosing metabolic acidosis in cattle and was used to differentiate metabolic acidosis caused by bicarbonate loss from that caused by accumulation of organic acid.(Constable et al., 1997; Stocker et al., 1999; Ewaschuk et al., 2003). Calculation of AG is based on the principle of electroneutrality and is calculated as the difference between routinely measured cations and routinely measured anions (Constable et al., 1997). Values in normal animals have a pronounced variability and range between 7.1 to 22.6 mmol/L (Shull, 1978; Stocker et al., 1999; Omole et al., 2001; Ewaschuk et al., 2003). Normal positive value of AG representing the charge on serum proteins, phosphate, and strong anions, which are not routinely measured. In our results, diarrhoeic calves showed a mean AG value of 17.06±6.14 mmol/L. This is in agreement with other reports that showed a mean AG value of 7.1±2.6 mmol/L in healthy calves and 23.4±7.7 mmol/L in diarrhoeic calves (Ewaschuk et al., 2003). Although the clinical utility of AG to predict blood L-lactate in sick calves has been questioned (Constable et al., 1997), increases in AG generally are

indicative of a gain of organic anions such as ketoacids, DL-lactate or uremic anions. (Stocker et al., 1999; Constable et al., 1997; Ewaschuk et al., 2003). Decrease in AG generally is indicative of laboratory error, hypoalbuminemia, and reduction in sTP concentration in adult cattle (Constable et al., 1997; Kraut and Madias, 2007). To the author's knowledge, this is the first report of a AG that seems to play an important role in the outcome of neonatal diarrhoea. One possible explanation for this is that the deviation from the normal value of the serum AG can reflect either clinicopathologic changes in neonatal calves with diarrhoea (e.g. > DL-lactate concentration; Ewaschuk et al., 2003) or poor passive immunity (e.g. < sTP concentration). This is not in agreement with Constable et al. (1997) and Lorenz (2004), who found that anion gap was not correlated with albumin or sTP and D-lactate had no impact on prognosis of neonatal diarrhoea. Further studies are necessary to determine if relationships between neonatal diarrhoea and AG, or between FPT and low AG value, exists and to further understand the physiological nature of these relationships.

The month of hospitalization had a moderate importance in the outcome of the disease. A possible explanation for this results is that during cold season hospitalization increases. Although this result is considerably influenced by the number of admissions in clinic, low temperatures, wind, and high levels of moisture act as stress factors for young calves and increase the susceptibility of calves to diarrhoea (Carroll and Forsberg, 2007). Neonatal calves are not able to effectively regulate their body temperature when exposed to extreme weather conditions. This may induce hypothermia or hyperthermia resulting in immune system impairment (Carroll and Forsberg, 2007). In addition, the absorption of Ig may be affected by the environment in which the calf is born. Extreme cold reduces the absorption of Ig by calves (Olson et al., 1980). The effects of ambient temperature outside the thermoneutral range for calves might involve direct effects on intestinal absorption and transport as well as the ability of the

calf to stand and nurse (Olson et al., 1980; Olson et al., 1981). Previous studies have revealed that although season was not significant in the final model, the odds of FPT were higher for dystocic calves that did not received a heat source during cold weather (Beam et al., 2009).

The results of this study have shown that acid base and electrolyte imbalance, vitality and dehydration score, rectal temperature and age have a low importance on the fate of diarrhoeic calves. Correlation between acid base disorder and clinical signs in NCD has been the objective of numerous studies. Variations in behaviour, posture and palpebral reflex are more closely correlated with increase of serum D-lactate concentrations than with decreases of BE (Lorenz, 2004; Lorenz et al., 2005; Gentile et al., 2008; Trefz et al., 2012b). As for the AG value, clinical presentation of the diarrhoeic calves could be related to the increase of D-lactate but this condition could not be correlated with the calves in this study because blood D-lactate concentration was not obtained. Previous study reported that calves with elevated D-lactate concentrations did not need additional specific therapy, as D-lactate concentrations regularly fell following correction of acidosis and restitution of body fluid volume (Lorenz, 2004a; Lorenz and Vogt, 2006). In our study, low importance of clinical and acid-base data in outcome of diarrhoeic calves is probably due to the good correction of acidosis and dehydration. Bicarbonate requirements, calculated using the formula: bicarbonate requirements (g) = body weight (kg) x BE (mmol/L) x 0.6 (L/kg) x 0.084 (g/mmol), have been criticized for the high rate of treatment failure (Lorenz and Vogt, 2006). Risk of failure to correct acidosis increased with D-lactate concentrations (Lorenz and Vogt, 2006). In according with Lorenz and Vogt (2006), our treatments failure were treated with further IV amount of bicarbonate so, in this study, lactic acidosis could be ruled out as as a possible factor associated with mortality of dairy calves with neonatal diarrhoea. Serum K concentration is usually high in diarrhoeic calves (Seifi et al., 2006; Trefz et al.,

2013a) and hyperkalemia results as was seen in our study. Hyperkalemia causes muscle weakness and potentially life-threatening cardiac arrhythmias (Trefz et al., 2013a). Recently Trefz et al. (2013b) have shown that dehydration is an important contributor to the pathogenesis of hyperkalemia and acidemia in neonatal calves with diarrhoea. In contrast with earlier findings (Seifi et al., 2006), in the present study logistic regression results show that K seem not to play an important role in mortality of dairy calves with neonatal diarrhoea.

Chapter three

Intravenous immunoglobulin transfusion in colostrum–deprived dairy calves

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Abstract

Immunoglobulin transfusion has been empirically employed in the management of therapy in FPT. The aim of this study is to establishing a dose of IgG needed to reach a protective levels (>10 g/L) in colostrum-deprived dairy calves that could be used as supportive therapy in FPT.

Twentyeight Holstein Friesian new born male calves were randomly assigned to either control or treated groups. Control calves received 4 L of high quality colostrum within 12 hours after birth. Treated calves received intravenously 61.63 ± 2.02 g of IgG in 2.67 ± 0.38 L of pooled plasma within 6 hours after birth. Serum IgG and serum total protein concentration were assayed immediately before and after (24h, 72h and 1 week after birth) plasma transfusion or colostrum ingestion. Serum IgG and serum total protein concentrations increased in both control and treated calves (>10 g/L). Mean concentration of sIgG and serum total protein, after colostrum ingestion or plasma transfusion, was higher in control calves than treated calves ($P = 2^{-16}$). Nine treated calves developed diarrhoea during the study and four died. None of the control calves showed signs of disease or died during the study. Although the dose of IgG used in this trial effectively provide an adequate sIgG concentration in colostrum–deprived calves (>10 g/L), control group calves had significantly lower morbidity and mortality rates compared to the plasma calves, suggesting that plasma transfusion alone is ineffective to provide a complete protection against neonatal disease.

Keywords: Calves, Colostrum, Failure of Passive Transfer, Immunoglobulin, Plasma Transfusion.

Introduction

Ruminants have a synepitheliochorial placenta which prevents transplacental transfer of maternal immunoglobulins to the fetus (Peter, 2013). Consequently, calves are hypogammaglobulinemic at birth (Chigerwe et al., 2008a). Colostrum plays the most important role in transmitting specific and aspecific passive immunity until the immune system of the newborn calves becomes functional (Godden, 2008). When adequate amounts of immunoglobulins are not absorbed, calves are defined FPT if either the calf s IgG or sTP concentration are less than 10 g/L and 52 g/L, respectively (Tyler et al., 1996). The estimated prevalence of FPT in U.S. dairy calves is 19% (Beam et al., 2009), in Canada 37% (Trotz–Williams et al., 2008) and in Australia 38% (Vogels et al., 2013). To the author's knowledge, there are not any published data on the prevalence of the FPT in Europe. Failure of passive transfer affects calf's health and survival way beyond the neonatal period (Tyler et al., 1999a; Virtala et al., 1999). Despite the significant progress made in understanding biology and mechanisms of FPT, the application of this knowledge into improved treatments of sick calves has been limited.

Immunoglobulin transfusion, through plasma administration, has been empirically recommended as a therapeutic intervention in calves with FPT (Weaver et al., 2000; Barrington and Parish, 2009). The first report of plasma transfusion in calves by Smith and Little is emerged in 1920s (Smith and Little, 1922). In this study the authors suggested that oral and parenteral administration of small doses of plasma can be protective in colostrum–deprived calves, although sIgG concentration after plasma administration was not determined. Anderson et al. (1987) showed that plasma transfusion of 20 mL/kg increased the concentration of sIgG in colostrum–deprived calves, albeit not to protective levels (sIgG <10g/L). Selim et al. (1995) evaluated the efficacy of hyperimmune plasma, and concluded that it was not superior to normal plasma or to no

intervention, in terms of calf morbidity and mortality. However, in the study by Selim et al. (1995) the passive transfer status of the calves before transfusion was not determined. Quigley and Welborn (1996) showed that a purified Ig preparation increased sIgG concentrations in colostrum–fed calves after IV or SC infusion, but this treatment had no effect on morbidity or mortality of neonatal diarrhoea. Recently Chigerwe and Tyler (2010) concluded that administration of 0.5 L of commercially available serum product did not provide adequate sIgG concentrations in neonatal Jersey and Jersey–cross calves with FPT, as shown by non–significant differences of sIgG concentrations between control and transfused calves.

The experimental data are rather controversial and there is no general agreement about the correct dosage of IgG in the treatment of FPT. A considerable amount of the revised literature failed to specify the effectiveness of transfused IgG to reach serum protective levels (>10 g/L). This was mainly due to the following reasons: 1) no information about the sIgG concentration pre and post plasma transfusion, 2) no information about the concentration of IgG in the transfused products or 3) injection of a small dose of IgG.

This study compared sIgG and sTP in a control group of calves feed with a high quality colostrum vs. a treated group of colostrum–deprived calves submitted to an IV administration of IgG by plasma transfusion for establishing a dose of IgG needed to reach a protective levels (>10 g/L) that could be used as supportive therapy in sick calves affected with FPT. Furthermore we have evaluated the differences between the two groups in terms of morbidity and the mortality.

Materials and Methods

Animals

A randomized clinical control trial was performed at the Clinic for Ruminants, Swine and Management (CRSM) of the Large Animals Veterinary Teaching Hospital, University of Milan. All procedures were approved by the ethical committee of the University of Milan.[1] Twenty–eight Holstein Friesian male calves, born of multiparous dairy cows from a single dairy farm were included in this study. The study was conducted between September and December 2013. After parturition, calves were immediately separated from their dam, weighed, identified, admitted to the CRSM, placed in individual hutches (1.8×1.2 m) with a straw litter and randomly assigned into two groups by a dedicated web site.[2] Control Group (CG) consisted of 14 calves (mean body weight of 44.6 kg) that received high quality bovine colostrum. The Plasma Group (PG) consisted of 14 colostrum–deprived calves (mean body weight of 45.4 kg), received IV fresh frozen plasma transfusion at 6–8 hours after birth.

Clinical procedures

CG calves received 4 L of colostrum in 2 separate feedings: the first feeding (2 L) occurred in less than 3 hours after birth, and the second feeding (2 L) occurred within 12 hours after birth. PG calves received 61.63 ± 2.02 g of IgG in 2.67 ± 0.33 L of plasma (see later). Plasma transfusion was performed via the jugular vein with 14 G IV catheter, using an intravenous line with filter (Transfusion set PQ 601–TS; Ferrari L. S.R.L., Verona Italy) Infusion of plasma was performed slowly (10 mL/kg/h) over the first 20 minutes. Monitoring for transfusion reactions included heart and respiratory rate, color of mucous membranes, and abnormal behavior (Chigerwe and Tyler, 2010). In the absence of an immediate transfusion reaction, the remainder of the plasma was transfused at 50 mL/kg/h. Blood samples from calves were collected at birth

before receiving colostrum or plasma transfusion (T_0), and at 24 h (T_1), 72 h (T_3), and at 1 week (T_7) of age. T_1 samples were performed after either transfusion or colostrum administration. Briefly, 10–mL blood samples were collected by jugular venipuncture into Vacutainer™ tubes with no additive. Blood was allowed to clot and then centrifuged at 20 °C for 10 minutes at 900 X g. Serum was transferred into two Eppendorf™ tubes, harvested and stored at –20 °C until analysis to determine sTP and sIgG concentration (see later). After colostrum or plasma transfusion, calves were fed with powdered milk substitute (3 meals/2L/day). Both groups were monitored by a daily clinical examination for three weeks.

Colostrum collection and storage

Colostrum used in this study was obtained two months before from cows from the same herd. Cows were milked within 1 hour after parturition. An aliquot of the first milking colostrum from each cow was collected from the milking bucket for refractometric determination (Rifrattometro PCE 0–32%; PCE Italia S.R.L., Capannori Italy) of BRIX–point–colostrum quality. If colostrum quality was adequate (≥22% of the BRIX 0:32 scale; Bielmann et al., 2010), 4 L were stored at -20 °C until administration.

Plasma collection and storage

Whole blood was collected from 20 multiparous, healthy adult dairy cows (age 4 to 7 years). Cows were from the same herd of the calves used in this study and whole blood was obtained two months before clinical trial. From each cow, 5 L of whole blood were collected according to standard techniques (Soldan, 1999) by jugular venipuncture, using a 12–gauge needle attached to a 450 mL (11 bags from each cow) double–bag closed–collection system (CPD–A 450; Laboratory Grifols Italy S.P.A, Vicopisano Italy). The closed–collection system consisted of

a primary bag that contained 63 mL of citrate phosphate–double dextrose solution as an anticoagulant and 1 empty satellite bag. Bags with whole fresh blood were immediately refrigerated at 4 °C and centrifuged at 3500 X g for 15 minutes at 4 °C. Centrifuge (Rotixa 500 Rs; Hettich Zentrifugen, Tuttlingen Germany) was set at rapid initial acceleration rate (run up 9) and free deceleration (run down 0). A plasma extractor (Plasma Extractor; Fenwal Inc, Mont Saint Guibert Belgium) was then used to generate 1 bag of plasma from each bags of whole blood. During plasma extraction an aliquot was collected from all plasma bags and used to assay IgG concentration with an ELISA catching system, as described below. Assuming that the bags of similar volume coming from the same donor cow had the same IgG concentration, an additional ELISA (Quigley, 2008) test was performed only for the aliquots sampled from the bags that had a consistent volume difference (>10 mL). The objective was to evaluate the discrepancies of IgG concentration caused by volume variability of single plasma bags and anticoagulant contained in the closed–collection bags system. The 11 plasma bags of the same donor cows were pooled and immediately frozen at -20 °C. After the ELISA test we pooled 14 set bags from donor cows with the highest IgG concentration. Plasma bags set were created in order to get 14 pre–set plasma bags for each calf, each containing approximately 62.5 g of IgG (as described below).

Plasma IgG dose prediction

According to the formula by Chigerwe and Tyler (2010), the estimation of total IgG amount transfused in PG calves was calculated as follows:

Total IgG amount in transfused plasma g = (post transfusion sIgG desired concentration g/L) x (BW kg x % L plasma volume)/(estimated sIgG pre transfusion g/L).

Assuming a plasma volume of 8.9 % of calf body weight (Quigley et al., 1998) 0.639 g/L mean pre treatment sIgG concentration in calves (Chigerwe et al., 2008a) 44.9 kg mean body weight at birth (Gianola et al., 1974) and 10 g/L of desired post transfusion total sIgG concentrations Tyler et al., 1996), estimation of total IgG amount transfused in PG group calves, was calculated as follows:

Total IgG amount in transfused plasma g = (10 g/L) x (44.9 kg x 0.089)/(0.639 g/L) = 62.5 g

Serum TP and sIgG titrations

The concentration of sTP was estimated using a standard quantitative colorimetric assay for serum and plasma (Total proteins quantitative colorimetric assay biuret method on serum and plasma; Biochemical Enterprise, Milano Italy), according to manufacturer's protocol. The colorimetric results were determined by a clinical chemistry analyzer (Roche Cobas Mira Classic; Hoffmann–La Roche, Basel Switzerland). The immunoglobulin concentrations in both calves serum and single donor cows plasma bags were analyzed by an ELISA catching system (Bovine IgG ELISA quantitation set; Bethyl Laboratories, Montgomery U.S.) (Quigley, 2008), as follows. Wells of 96–well microtitre plates were coated for 1 hour at room temperature with 100 μl/ well of capture affinity purified antibody (sheep anti–bovine IgG) diluted 1:100 in coating buffer (carbonate–bicarbonate buffer 0.05 M, pH 9.6). After 5 washes with TBST (Tris Buffered Saline [TBS] 50 mM pH 8.0, supplemented with 0.05% Tween 20), the wells were post–coated with 200 μl/well of TBST for 30 minutes, washed 5 times with TBST, and incubated with either serum samples from calves or plasma samples from donor cows, diluted 1:200,000 and 1:100,000 in TBST, respectively (these dilutions were determined by previous preliminary set up experiments). Seven, two–fold dilutions in PBST of purified reference bovine antibodies were used to perform a standard curve (500 to 7.8

ng/ml). The wells were filled with 100 μl of diluted sample or reference serum in duplicate for 1 h, washed 5 times, and then incubated for 1 h with 100 μl/well of secondary antibody (sheep anti–bovine IgG HRP (Horseradish Peroxidase)–conjugate, diluted 1: 100,000). After 5 washes, colorimetric reaction was developed by adding 100 μl/well of substrate–chromogen (H_2O_2 and TMB, 3.3', 5.5' tetramethylbenzidine) for 15 minutes in the dark, and then stopped by adding 100 μl/well of stop solution (sulfuric acid 0.18 M). The colorimetric reaction was determined by absorbance at 450 nm with an ELISA micro–plate reader (Titertek Multiskan; Labsystem, Vantaa Finland) expressing the result as optical density. All the results were expressed as grams of IgG per L (g/L) by interpolating the absorbance results with the serum standard curve.

Statistical analysis

The increase in sIgG and sTP concentrations from T_0 to T_1 was calculated for control and treated calves and subsequently compared using a 2–sample t–test. A linear mixed model was used to compare at different time points, sIgG or sTP concentrations from control and treated calves. Three time points were considered: 24 (T_1), 72 hours (T_3) and 1 week (T_7) after colostrum/plasma administration. The fitted mixed model included the fixed effects of treatment (colostrum/plasma administration) time, their interaction and a random calf effect. Least–square means for fixed effects and their interaction were obtained. All response variables were tested for normality using the Shapiro–Wilk test. Proportions of calves who developed diarrhoea during the experiment were compared by chi–square analysis. All statistical analyses were performed using the R programming environment. The R packages *lme4* and *lmerTest* were used for fitting the mixed model and to estimate the least–square means, respectively.

Results

The total volume of collected plasma was 60.2 L distributed in 126 bags. Single bag volume ranged from 265 to 380 mL. Total IgG amounts in individual plasma bags ranged between 4.9 and 10.1 g with an average IgG concentration of 7.87 $\pm$ 1.73 g. The average IgG dose transfused was 61.63 $\pm$ 2.02 g corresponding to a mean transfused plasma volume of 2.67 $\pm$ 0.38 L. A total of 8.2 $\pm$ 1.4 bags of plasma were given to each calf. Neither of PG calves showed transfusion reaction and the transfusion lasted for a maximum of 3 hours. Results of sIgG and sTP concentration are summarized in Table 6. At T_0 eleven (11/14) CG calves and six (6/14) PG calves had a detectable sIgG concentration with a mean value of 2.62 $\pm$ 1.97 and 1.52 $\pm$ 2.40 g/L, respectively. The range of variation was 0.97 to 6.85 g/L and 1.94 to 6.58 g/L. At T_0 sTP concentration in CG calves and PG calves ranged from 36 to 46 g/L and from 33 to 41 g/L with an average of 41.14 $\pm$ 3.79 and 36.64 $\pm$ 2.87, respectively. At T_1, sIgG and sTP concentrations increase in both CG and PG calves. Mean increase between T_0 and T_1 in sIgG were 29.44 $\pm$ 7.06 g/L for CG calves and 17.46 $\pm$ 7.06 g/L for PG calves (Figure 4). The t–test used to compare those results was statistically significant ($P = 1.3 \times 10^{-4}$). Mean increase in sTP between T_0 and T_1 were 19.64 $\pm$ 7.16 g/L for CG calves and 13.28 $\pm$ 5.25 g/L for PG calves (Figure 5). The t–test used to compare the increase in sTP between T0 and T1 was statistically significant ($P = 0.0132$). Results from mixed model analysis of variance for sIgG concentrations over time confirmed the significant effect of treatment, time and their interaction (Table 7). Calves fed colostrum had significantly higher serum IgG concentrations compared with PG calves although CG and PG calves showed an average sIgG value >10g/L from T_1. Results from mixed model analysis of variance for sTP concentrations over time confirmed the significant effect of treatment, time and their interaction (Table 8). The control group calves showed over the entire period higher sTP concentrations than PG calves.

Nine (9/14) PG calves developed diarrhoea during the study. Calves that scoured were treated with an intravenous fluid therapy (Berchtold, 2009). The four sick PG calves died at 5, 11, 12, 16 days of age, respectively. Necropsy indicated that the primary cause of mortality was enteritis complicated by *Escherichia coli* septicemia. None of the CG calves showed signs of disease or died during the study. The Pearson's Chi–squared test performed on those data was significant ($\chi2$ = 10.4795; P < 0.001; degrees of freedom = 1).

Discussion

Passive transfer of immunity is a critical factor and an important concern for the management of neonatal calf health. FPT is associated with increased risk of disease, mortality, and reduced performance in adult cattle (Robinson et al., 1988; DeNise et al., 1989; Wells et al., 1996). Moreover, diseases of sick calves with FPT often have a poor prognosis (Fecteau et al., 1997; Tyler et al., 1999b). Absorption of colostral Ig by the intestine of calves decreases quickly, and completely ceases approximately 24 h after birth, limiting the time available for the veterinarians to administer colostrum therapeutically (Berge et al., 2009). Therefore, transfusion of a large dose of IgG could play a key role for treating calves with FPT.

The present study was designed to determine a standardized dose of IgG needed to reach a protective level (>10 g/L) which could be used as supportive therapy in calves with FPT. Results showed that $\approx$ 62 g of IV plasma IgG increased sIgG concentrations to levels considered adequate for passive transfer of immunoglobulins ($\geq$10 g/L; Tyler et al., 1996). To the author's knowledge, this is the first report of a plasma transfusion that successfully replenished the sIgG pool of colostrum–deprived calves to satisfactory levels. Previous studies described the ineffectiveness of plasma transfusion in achieving adequate sIgG

concentrations in colostrum–deprived calves (Anderson et al., 1987) or in calves with FPT (Chigerwe and Tyler, 2010). In the study by Anderson et al. (1987) colostrum–deprived calves received plasma intravenously or intraperitoneally at 20 mL/kg. However, the concentration of IgG in the pooled plasma used in this study was not determined. Recently, Chigerwe and Tyler (2010) concluded that administration of 0.5 L of commercially available serum product did not provide adequate sIgG concentrations in calves with FPT. In that study, transfused calves received a total IgG dose of ≈ 8 g. The anticipated increase in sIgG concentration, calculated using a plasma estimate equal to 5% of body weight, was 11.32 g/L, while it only increased up to 7.4 g/L. Because of this discrepancy, the authors concluded that the predicted concentration of sIgG after transfusion is strongly affected by the estimate of plasma volume (Chigerwe and Tyler, 2010). Some authors suggested that administration of IgG supplements does not achieve protective concentrations of sIgG. This is due to the underestimation of the required supplementation dose (Crawford et al., 2003), or because transfused IgG, especially when given at low doses, can leak into extravascular pools, decreasing the concentrations of sIgG (Quigley and Welborn, 1996). In contrast to earlier findings (Chigerwe and Tyler, 2010), in the present study, after plasma transfusion, PG calves showed mean sIgG concentration higher than the estimated concentration. There are several possible explanations for this result. Discrepancy may be due to the possible variability of plasma calves volume. In this study, the plasma volume of transfused calves was affected by colostrum deprivation. Previous studies reported that blood volume increases from 5.3% of BW at birth to 6.5% of BW at 1 d of age after colostrum ingestion (Mollerberg et al., 1975). McEwan et al. (1968) reported an increase in plasma volume from 6.6 to 9.3% of BW after calves were fed colostrum during the 1st d after birth. Quiqley et al. (1998) showed that an 8.93% plasma volume was appropriate for colostrum fed calves

24 h after birth. However, the plasma volume based on BW showed large variability (Quiqley et al., 1998). Using a lower percentage of body weight to predict sIgG concentration would have mitigated the overestimation effect, producing results more comparable to observed data. On the other hand, using a larger blood volume obviously reduced the risk of not attaining the required IgG dose. Another possible explanation for this results is that calves used in this study had a precolostral sIgG concentrations greater than expected. Six (6/14) PG calves had a detectable sIgG concentration and mean $\pm$ SD pre–transfusion serum IgG concentration was 1.5 $\pm$ 2.4 g/L. In accordance with our results, recent studies demonstrated that a high precolostral concentrations of IgG is more common than reported in the past (Chigerwe et al., 2008a; Fux and Wolf, 2013). High precolostral IgG concentration can be associated with a transfer of maternal antibody across the placenta for unknown reasons or with an undetermined transplacental infection (Chigerwe et al., 2008a; Fux and Wolf, 2013). Although mean sIgG concentration was higher than expected, the sIgG concentrations were still higher in CG than in PG. These results may be explained with the large amount of IgG absorbed with colostrum. In our study CG calves received 4 L of high quality bovine colostrum. Break point of 22% Brix could be considered an acceptable cut–off point for high–quality colostrum (>50 g of IgG/L; Bielmann et al., 2010). Assuming that colostrum IgG–apparent efficiency of absorption ranged between 20 and 30% (Quigley and Drewry, 1998) CG calves received a large amount of IgG than PG calves. In accordance with the results of sIgG, CG calves had significantly higher sTP than PG calves. However, contrary to sIgG concentration, the average of sTP in PG calves resulted <52 g/L indicating a poor passive immunity (Tyler et al., 1996). This finding was unexpected and it could be the consequence of a greater protein amount in the administered colostrum (compared to plasma), and to an overall greater efficiency of intestinal absorption of the colostrum proteins (Foley and

Otterby, 1978; Kehoe et al., 2007; Poulsen et al., 2010). In addition to a large amount of proteins, colostral growth factors alter the development of digestive and absorptive capacities of neonatal gastrointestinal tract and causes considerable metabolic changes in calves (Blum and Hammon, 2000). Rauprich et al. (2000) showed that serum urea concentration was high in calves fed with milk–based formula with small amount of IGF–I than maternal colostrum. These findings suggested that colostral growth factors exerted an anabolic effect and thereby reduce plasma urea concentration (Blum and Hammon, 2000). Therefore, colostrum intake increased protein metabolism and affected sTP concentrations (Blum and Hammon, 2000; Zanker et al., 2000). Contrary to expectations, sTP cut–off point used to predict which calves had adequate passive immunity ($\geq$52 g/L), cannot be used in colostrum–deprived calves subjected to plasma transfusion. This study demonstrated that a sTP concentration <52 g/L did not suggest failure of passive transfer, because after transfusion of $\approx$ 62 g of IgG, sIgG concentration in PG calves was >10g/L.

Nine PG calves developed diarrhoea and 4 calves died during the study. These results are consistent with other studies and suggest that Ig transfusion into peripheral circulation might not decrease the incidence of enteric disease in FPT calves (Quigley and Welborn, 1996). In fact, in addition to IgG, colostrum contains other important substances that are involved in natural host defense system against local invading microorganisms (Przybylska et al., 2007). Colostrum passive mucosal immunity factor, antimicrobial factor and growth factor are implicated in the development of neonatal immune system and in the prevention of enteric diseases in young calves (Corley et al., 1977; Saif et al., 1985; Besser et al., 1988a,b; Baumrucker et al., 1994; Bird et al., 1996; Pakkanen and Aalto, 1997; Elfstrand et al., 2002). We speculate that colostrum–deprived calves of our study, although transfused with plasma, received fewer amounts of other components (growth hormones, cytokines, maternal leucocytes,

antimicrobial factors) commonly found in colostrum, leading to higher incidence of enteric diseases and mortality.

Chapter four

Conclusion

The results of chapter 2 demonstrate that the sTP concentration represents a major factor associated with mortality of dairy calves with neonatal diarrhoea. Therefore, colostrum management practices seem to play a significant effect on the risk of treatment failure in naturally infected diarrhoeic dairy calves. In the future, more clinical studies are needed to further correlate sTP concentration and the successful treatment of NCD, in order to determine if the use of this simple parameter allows predicting outcome with a good probability of exactitude. This could assist the veterinarian for a more accurate prognosis, for a more accurate choice of a therapeutic protocol and to estimate the cost of the treatment required for a successful outcome.

Possible future studies may further evaluate the role of AG in mortality of dairy calves with neonatal diarrhoea.

Furthermore we speculated that calving season modification, when environmental conditions are more favorable, or special care required to reduce cold environmental temperature risk, are needed to improve outcome of NCD.

Results of chapter 3 demonstrate that although IV infusion of ≈ 62 g of plasma IgG was an effective method for providing an immunoglobulin mass necessary to achieve adequate transfer of passive immunity in neonatal colostrum–deprived calves, calves fed with colostrum had significantly higher sTP and sIgG concentrations than transfused calves, and lower incidence of enteric diseases, suggesting that plasma transfusion does not represent a valid alternative to a good–quality colostrum administration to reach a complete transfer of passive immunity and protection against neonatal disease in colostrum-deprived dairy

calves. Because these findings suggest that $\approx$ 62 g of plasma IgG can provide a source of Ig for neonatal dairy calves after closure of the gut, future trials should assess the impact of high doses of IgG in terms of outcome in sick calves with failure of passive transfer focusing on possibility of identifying the potential dose of IgG adapted for each individual FPT calf for example through simple refractometric determination of sTP.

The results of data presented demonstrate that good practices of colostrum management are often implicated in the success of the treatment for NCD. Despite this, failure of passive transfer of immunity is difficult to correct.

Table 1. Guide to assessing the degree of vitality based on posture, behaviour and suckling reflex of the calf.

Vitality score	Clinical signs
5	Stand without assistance, strong suckling reflex, curious, alert
4	Standing up after encouragement, dull, weak suckling reflex
3	Standing after lifting, "drunken" gait, weak or absent suckling reflex
2	Permanent sternal/costal recumbency, absent suckling reflex
1	Lateral recumbency, coma

Table 2. Guide to assessing the degree of dehidration based on posture, behaviour, enophthalmos, skin tenting and extremities temperature.

Dehydration score	Clinical signs	Loss of body water (%)
0	None	0%
1	Mildly depressed, tight skin	~5%
2	Sunken eyes, tight skin, dry mouth and nose, but still standing.	~10%
3	Severe sunken eyes, cold ears, legs and oral cavity, unable to stand	~15%

Table 3. Mean value of age, clinical variables, results of blood analysis of all calves included in the study.

Predictors	N	Mean	SD.	Min	Max
Age	131	10.01	5.30	1	30
sTP	131	5.31	1.33	1.17	8.71
Temp	131	38.18	1.45	33.30	40.80
S_Dis	131	1.80	0.74	0	3
S_Vit	131	2.61	1.08	1	5
pH	131	7.18	0.15	6.84	7.51
pCO2	131	44.14	10.89	20.00	77.00
HCO3	131	17.35	7.73	4.20	39.30
BE	131	-10.46	9.34	-27.20	12.80
Na	131	132.01	14.33	106	184
K	131	5.62	1.55	1.80	9.60
Cl	131	102.26	15.34	5	146
AG	131	17.06	6.14	-0.10	31.60
Ht	131	33.35	8.74	17	63
Hb	131	11.04	3.06	5.60	21.10

sTP, serum total protein; Temp, rectal temperature °C; S_Dis, dehydration score; S_Vit, vitality score; pH, blood pH; pCO2, partial pressure of CO_2; HCO3, blood bicarbonate concentration; BE, Base Excess; Na, blood sodium concentration; K, blood potassium concentration; Cl, blood chloride concentration; AG, Anion Gap; Ht, haematocrit; Hb, hemoglobine; N, number of calves included; SD, standard deviatiom; Min, minimal value reported; Max, maximum value reported.

Table 4. Success and failure of therapy among the months.

Months	Therapy Failures	Successfully Treated
1	4	11
2	9	17
3	5	8
4	1	2
5	3	4
6	2	4
7	4	9
8	2	4
9	2	7
10	4	6
11	4	6
12	5	8

1, January; 12, December.

Table 5. Accuracy, Sensitivity and Specificity for *Training* and *Testing* data.

Dataset	Accuracy	Sensitivity	Specificity
Training	0.8	0.83	0.74
Testing	0.69	0.74	0.58

Table 6. Mean ± SD of sIgG and sTP for CG calves and PG calves at different blood samples time.

		T_0	T_1	T_3	T_7
CG calves	sIgG ± SD (gr/L)	2.62 ± 1.97	32.06 ± 6.63	30.32 ± 6.45	28.17 ± 8.14
	sTP ± SD (gr/L)	41.14 ± 3.79	60.78 ± 7.39	61.00 ± 5.44	57.85 ± 7.17
PG calves	sIgG ± SD (gr/L)	1.52 ± 2.40	18.98 ± 5.89	19.98 ± 6.26	18.17 ± 6.50
	sTP ± SD (gr/L)	36.64 ± 2.87	49.92 ± 5.58	50.78 ± 5.04	46.46 ± 7.12

CG, control group; PG, plasma group; sIgG, serum gamma immunoglobulin; sTP, serum total protein; T_0, blood samples collected at birth before receiving colostrum or plasma transfusion; T_1, blood samples collected at 24 h after treatment (colostrum/plasma transfusion); T_3, blood samples collected at 72 h after treatment (colostrum/plasma transfusion); T_7, blood samples collected 1 week after treatment (colostrum/plasma transfusion).

Table 7. Results from mixed model analysis of variance for sIgG concentrations over time.

| Effect	Estimate	SE	P value
Treatment			
CG calves	30.19	1.52	2^{-16}
PG calves	18.99	1.53	2^{-16}
Time			
T_1	25.53	1.26	2^{-16}
T_3	25.15	1.26	2^{-16}
T_7	23.09	1.18	2^{-16}
Treatment*Time			
CG calves T_1	32.06	1.78	2^{-16}
PG calves T_1	18.98	1.78	2^{-16}
CG calves T_3	30.32	1.78	2^{-16}
PG calves T_3	19.98	1.78	2^{-16}
CG calves T_7	28.17	1.78	2^{-16}
PG calves T_7	18.17	1.82	2^{-16}

CG, control group; PG, plasma group; sIgG, serum gamma immunoglobulin; sTP, serum total protein; SE, standard error T_0, blood samples collected at birth before receiving colostrum or plasma transfusion; T_1, blood samples collected at 24 h after treatment (colostrum/plasma transfusion); T_3, blood samples collected at 72 h after treatment (colostrum/plasma transfusion); T_7, blood samples collected 1 week after treatment (colostrum/plasma transfusion).

Table 8. Results from mixed model analysis of variance for sTP concentrations over time

| Effect	Estimate	SE	P value
Treatment			
CG calves	59.88	1.40	2^{-16}
PG calves	49.98	1.41	2^{-16}
Time			
T_1	55.36	1.20	2^{-16}
T_3	55.89	1.20	2^{-16}
T_7	52.04	1.21	2^{-16}
Treatment*Time			
CG calves T_1	60.78	1.70	2^{-16}
PG calves T_1	49.92	1.70	2^{-16}
CG calves T_3	61	1.70	2^{-16}
PG calves T_3	50.78	1.70	2^{-16}
CG calves T_7	57.85	1.70	2^{-16}
PG calves T_7	46.46	1.74	2^{-16}

CG, control group; PG, plasma group; sIgG, serum gamma immunoglobulin; sTP, serum total protein; SE, standard error T_0, blood samples collected at birth before receiving colostrum or plasma transfusion; T_1, blood samples collected at 24 h after treatment (colostrum/plasma transfusion); T_3, blood samples collected at 72 h after treatment (colostrum/plasma transfusion); T_7, blood samples collected 1 week after treatment (colostrum/plasma transfusion).

Figure 1. Correlation between variables. Increasing size and intensity of the staining of the ring, increasing the correlation between the two variables. Blue ring represents a proportional correlation, red ring represents inversely proportional correlation.
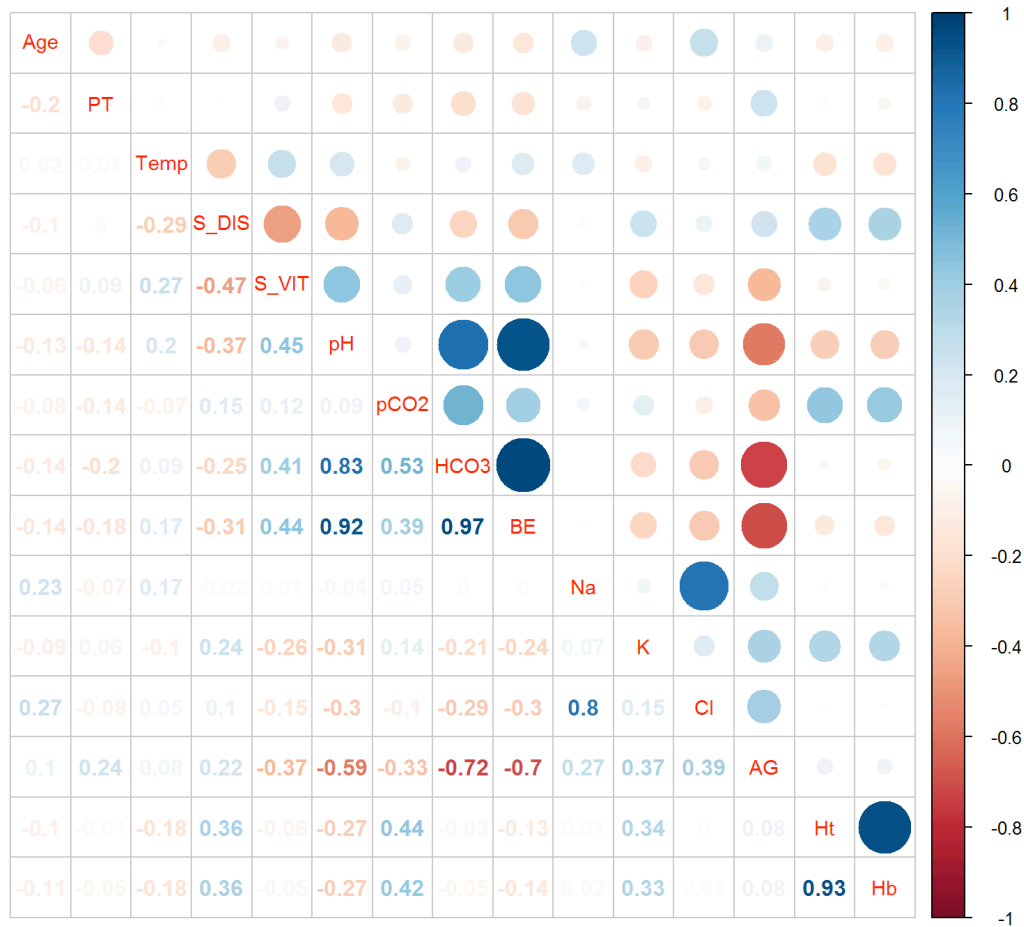
The strongest correlation were observed between HCO3 and BE (r= 0.97) and Ht and Hb (r= 0.93). Ht and BE predictors were not included in the final model.

Figure 2. Results of a logistic regression model predicting the importance of each variables having on outcome of neonatal diarrhoea
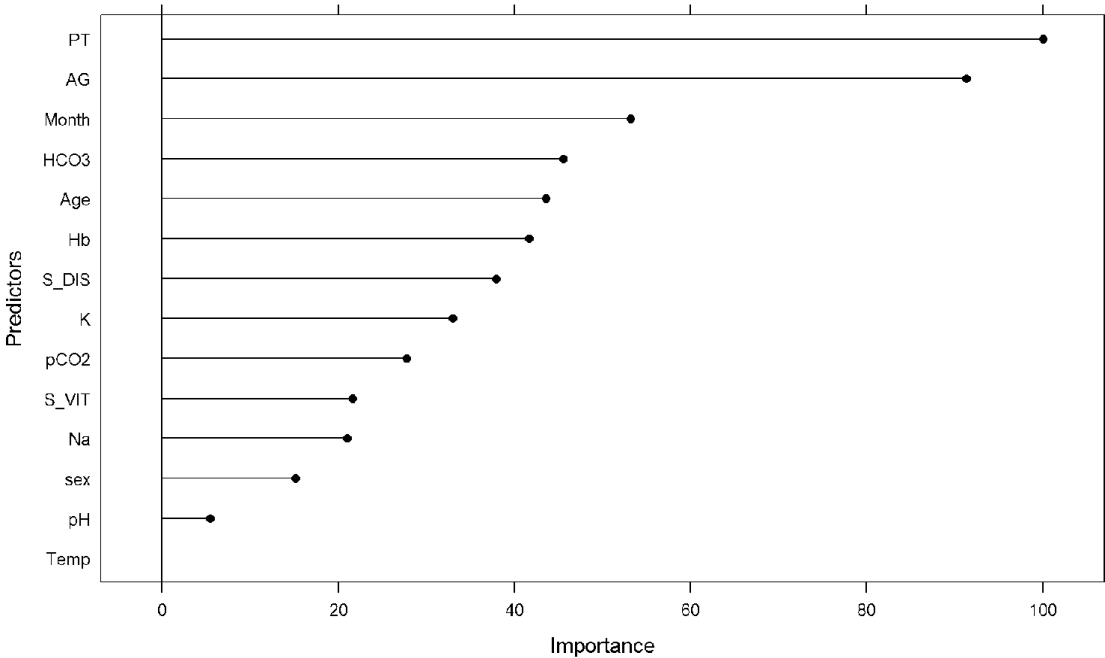
PT, serum total protein; AG, Anion Gap; Month, month of hospitalization; HCO3, blood bicarbonate concentration; Age, age in days; Hb, hemoglobine; S_Dis, dehydration score; K, blood potassium concentration; pCO2, partial pressure of CO2; S_Vit, vitality score; Na, blood sodium concentration; pH, blood pH; Temp, rectal temperature °C.

Figure 3. The relationship between survived/deceased calves and their sTP concentration
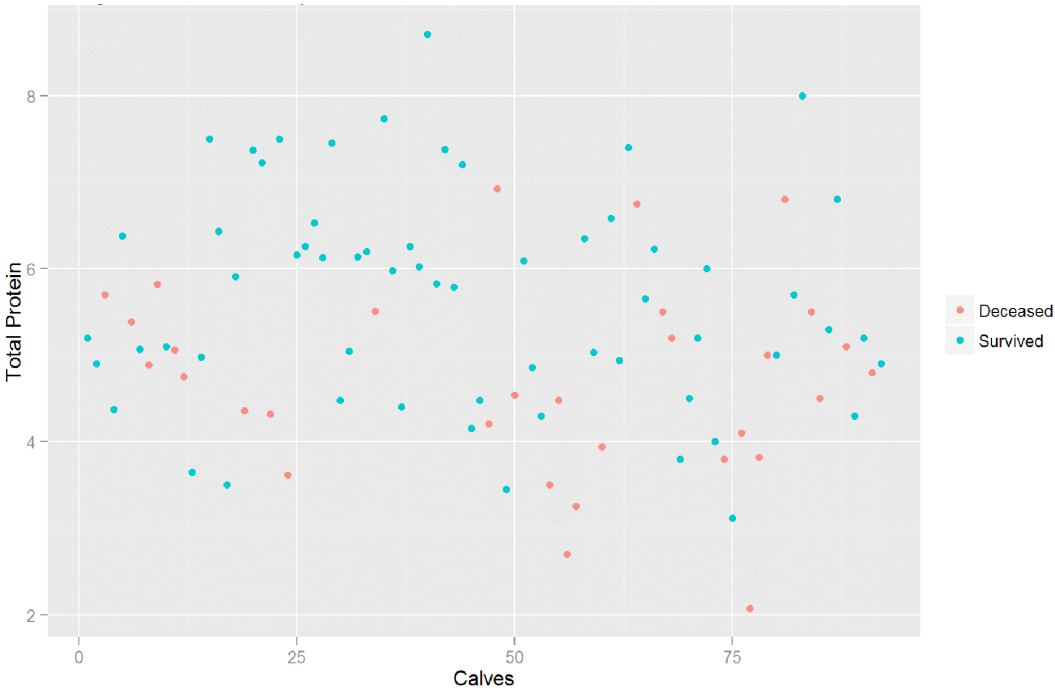
Figure 4. Average increase of sIgG concentrations in control group calves (CG calves) and plasma group calves (PG calves) from T_0 (before treatment) to T_1 (24 h after treatment).
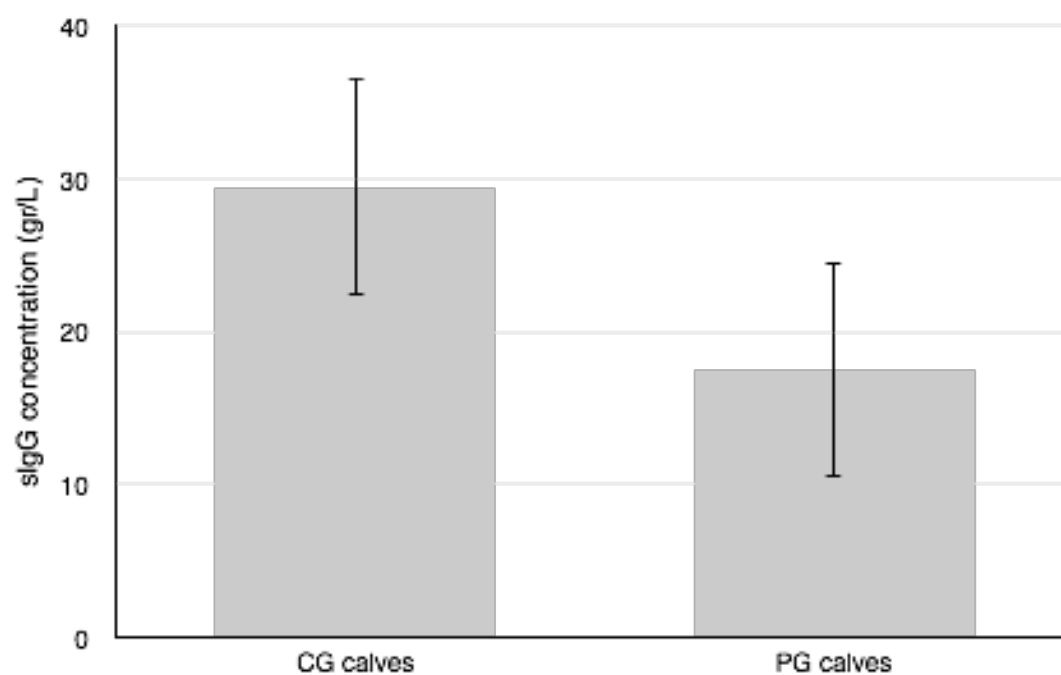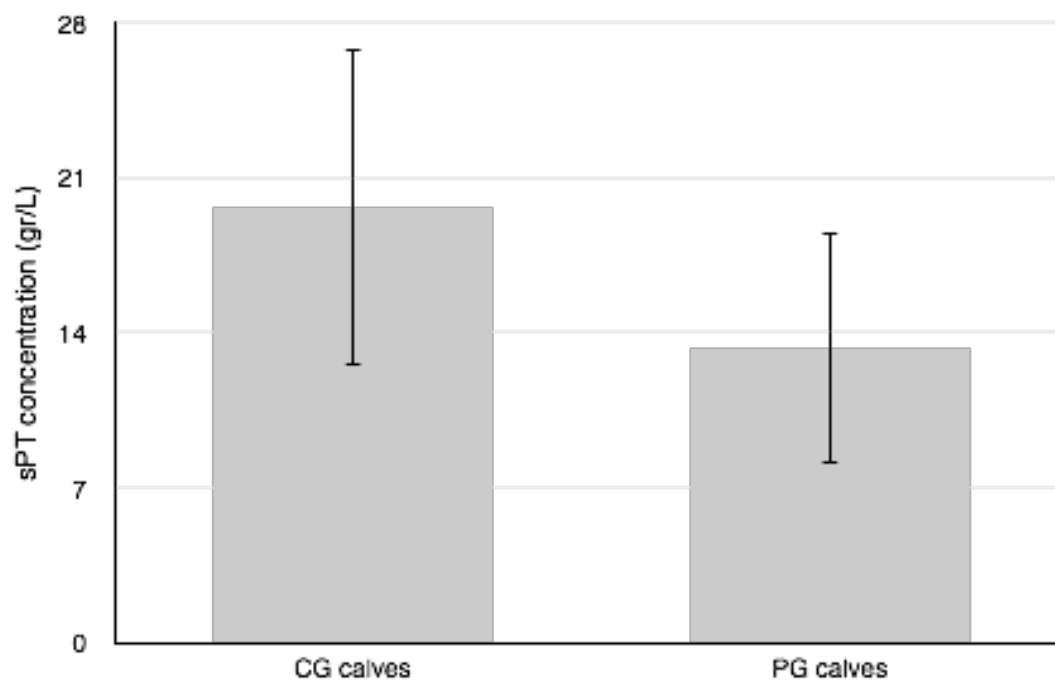
Figure 5. Average increase of sTP concentrations in control group calves (CG calves) and plasma group calves (PG calves) from T_0 (before treatment) to T_1 (24 h after treatment).

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