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Evaluation of passive transfer, apparent efficiency of absorption and health in dairy calves

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Evaluation of passive transfer, apparent efficiency of absorption and health in dairy calves

Passiv överföring av råmjölksantikroppar, absorptionseffektivitet och koppling till hälsa hos mjölkraskalvar

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SUMMARY

Calves are born without preformed antibodies and are dependent on colostrum to acquire passive transfer of immunity from their mother. Failure of passive transfer (FPT) is a contributing factor to increased calf mortality and morbidity, and hence of economic importance to the producer. The level of passive transfer (PT) is associated with future health and productivity even in adult age.

Several factors that influence the level of passive transfer in the calf are well studied, especially the importance of colostrum quality. The role of efficiency of absorption in the calf is less well understood. The aim of this study was to determine serum immunoglobulin G (S-IgG) by an enzyme-linked Immunosorbent assay (ELISA) newly established in our lab, estimate apparent efficiency of absorption (AEA) of 803 calves from two Swedish farms and investigate how they affected calf health and FPT.

An ELISA protocol was established in our laboratory to determine S-IgG concentration of 700 calves at 2-7 days of age and AEA was calculated. The ELISA S-IgG values were correlated to serum total protein (STP) measured by an optic refractometer (n=224) with $r=0.75$ ($p<0.0001$) for Spearman's rank correlation coefficient. Despite a good correlation, the ELISA was likely to overestimate S-IgG concentration and this probably affected the proportion of FPT. Proportion of FPT was clearly lower than previous study partly using the same samples (De Haan, 2018). The FPT for Lövsta was found to be 9.5% and for Röbbäcksdalen 30.4%, compared to previously 16% and 44.7%, respectively.

AEA range and mean of the farms were very similar, but there was a greater difference in mean S-IgG between the farms. Mean AEA of calves classified in our study to have FPT was significantly lower than the total average AEA of calves from the same farm. Surprisingly, both mean S-IgG concentration and AEA were significantly lower for healthy calves than unhealthy calves. This comparison included only calves from Lövsta, which had relatively few and mild cases of illness. Röbbäcksdalen did not have sufficient data available to be included. Birth weight and amount of IgG fed first meal were not significantly correlated to AEA in this study.

SAMMANFATTNING

Placantan bland hov- och klövdjur tillåter inte passage av stora molekyler så som antikroppar. Kalvar måste därför få det passiva skyddet via råmjölken som är som rikast på antikroppar precis efter kalvning. En fullgod passiv immunitet (PT) hos kalven minskar risken för, och svårighetsgraden hos, eventuella infektioner från omgivningen och har också visat sig ha positiva effekter längre fram i livet med bättre hälsa och kortare tid till första insemination. Det finns därför ett intresse att optimera andelen kalvar med god passiv immunitet då det finns indikationer att det skulle spara lidande vid sjukdom, antimikrobiella behandlingar och ge bättre produktivitet hos djuren.

Flera faktorer påverkar graden av passiv immunitet som kalven uppnår. Några av de mer väldokumenterade faktorerna är kvalitet av råmjölken som ges kalven såväl som tid och mängd av första mål råmjölk. Effekten av kalvens egen absorptionsförmåga på den passiva immuniteten är mindre klarlagd. Det främsta målet i den här studien var att anpassa en kvantitativ enzyme-linked immunosorbent assay (ELISA) för att direkt mäta koncentrationen av IgG från kalvserum och tillsammans med tillgänglig data estimerade effektiviteten i upptaget per individ, kallat apparent efficiency of absorption (AEA), och relatera till graden av sjukdom och om det finns indikation på samband med graden av Failure of Passive Transfer (FPT).

Serumkoncentration av immunoglobulin G (S-IgG) bestämdes i 700 prover från kalvar i åldern två till sju dygn och från två olika gårdar. Ett värde på AEA kunde därefter beräknas där data var komplett. Då flera prover analyserats med optisk refraktometer avseende serum-totalprotein (STP) som en del av en tidigare studie, kunde även korrelationen mellan de olika metoderna uppskattas. Korrelationen var $r=0,75$ ($p<0.0001$, för Spearman's rank korrelationstest). Vid jämförelse över hur analysmetoderna klassificerade fall av FPT, var andelen FPT betydligt lägre vid mätning med ELISA jämfört med optisk refraktometer. Andelen FPT för Lövsta var 9,5 % och 30,4 % för Röbbäcksdalen i denna studie jämfört med tidigare studie med delvis samma material 16,0 % and 44,7 %. Detta hör troligtvis ihop med att ELISA visade tendens att överestimera koncentrationen S-IgG.

Fördelningen och medelvärde på AEA var mycket lika mellan de olika gårdarna trots att de skiljde sig i medel S-IgG. Kalvar klassificerade som FPT med vår ELISA visade sig ha signifikant lägre genomsnittlig AEA jämfört med kalvar med S-IgG över cutoff på 10g/L. Överraskande visade sig gruppen av kalvar med sjukdom ha signifikant högre AEA och S-IgG än gruppen av sjuka kalvar. Denna jämförelse inkluderade enbart kalvar från Lövsta då kalvar från Röbbäcksdalen inte hade tillräckligt med data för att inkluderas. Kalvarna på Lövsta hade under perioden få och milda fall av sjukdom. Analys av korrelation mellan AEA och födelsevikt och AEA och massa IgG given vid första målet visade inget linjärt samband med statistisk signifikans.

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INTRODUCTION

Due to the placenta in ruminants, calves cannot receive any maternal antibodies in utero and are born agammaglobulinemic. Because of this, the neonatal ruminant is dependent on colostrum to supply it with the protective immunoglobulins (Ig). Ig combined with other immune stimulatory factors in colostrum can ameliorate severity, and counteract development of some infections (Weaver *et al.*, 2000; Godden, 2008).

The transfer of maternal antibodies to the calf is termed passive transfer and can fail for several reasons (failure of passive transfer, FPT). Some reasons are relatively well characterized such as a great variation in concentration of Ig in colostrum recognized between individual cows (Moore *et al.*, 2005; Liberg, 2000) and the calf not ingesting enough colostrum before intestinal permeability to macromolecules is lost (Godden 2008). But some cases of FPT cannot be explained by any of the well-known causes and calves are sometimes low in serum IgG despite good quality colostrum fed at appropriate age. Differences in efficiency in absorption (AEA) of Ig from colostrum is one factor suspected to contribute to FPT and variation in AEA is reported in several studies (Halleran *et al.*, 2016; Quigley & Drewry, 1998; Osaka *et al.*, 2014).

The aim of this study was to determine S-IgG by an ELISA newly established in our lab, estimate AEA and measured S-IgG in relation to calf health and FPT.

LITERATURE REVIEW

Neonatal immunity – the situation at birth

At birth, the neonate is transferred from a sterile environment in the uterus and is immediately exposed to microorganisms of the outside world, whereof some are pathogenic. Young domestic large animals are capable of mounting both innate and adaptive immune responses at birth although any adaptive immune response in the neonate will be a slow process since it is a primary response (Nonnecke *et al.*, 2012; Tizard, 2013). The results by Nonnecke *et al.* (2012) suggests that young calves are capable of vigorous humoral response in absence of protective maternal antibody. The response to a novel antigen, ovalbumin, (not included in the maternal antigenic experience), was comparable between colostrum deprived and colostrum fed calves in this study.

Ruminants have a syndesmochorial placenta where transplacental passage of large molecules such as immunoglobulins is prevented (Tizard, 2013). Colostrum is the only source of Ig and other large molecules for newborns in those species (Godden, 2008).

Passive transfer

Passive transfer is the process of absorption of maternal Ig from colostrum across the small intestine during a short period of time after birth (Godden, 2008). In calves with less than 10 g/L of IgG in their serum after closure (24-48h), this transfer is regarded as insufficient to protect from illness due to microorganisms and this is defined as Failure of passive transfer (FPT) (Liberg, 2000). Assuring early and adequate ingestion of colostrum by the calf is widely recognized as an important intervention to promote health and survival of neonatal calves (Godden, 2008). Immunoglobulins of several classes, A, D, G and M are absorbed (Tizard, 2013; Baintner, 2007).

Maternal passive immunity offers protection from pathogens from the mother's immune experiences (the microorganisms encountered in her environment) (Nonnecke *et al.*, 2012). Should the neonate encounter a pathogen not familiar to the maternal acquired immunity, it develops a response similar to that of a calf deprived of passive transfer (Nonnecke *et al.*, 2012).

In addition to immunoglobulins and nutrition, the colostrum contains immunologically active cells and mediators. Labeled maternal leukocytes in colostrum has been demonstrated in circulation of neonatal pigs, and absorption of immunologically active mediators may enhance the neutrophil function in the neonate (Weaver *et al.*, 2000).

Reduced post-weaning mortality, improved weight gain, improved feed efficiency, reduced time to first insemination, improved milk production during first and second lactation, and reduced tendency for culling during first lactation are among the long-term benefits associated with successful passive transfer (Godden, 2008; Furman-Fratczak *et al.*, 2011).

Failure of passive transfer

Failure of passive transfer is not at disease itself, but makes the young more susceptible to infection (Weaver *et al.*, 2000). FPT (serum IgG concentration below 10 g/L or serum total protein (STP) <55g/L) has been associated with increased health issues such as bacterial omphalitis, respiratory tract disease and diarrhea (Nilsson, 2015; Furman-Fratczak *et al.*, 2011). Donovan *et al.*, (1986) observed that calves dying from of infectious disease had lower STP than average. Calves with >15 g/L IgG in serum avoided respiratory tract infection (Furman-Fratczak *et al.*, 2011)

Liberg (2000) reported a prevalence of FPT in Swedish dairy calves of 14%, while a more recent study, showed a prevalence of 23% FPT (de Haan, 2018). Worth mentioning is that the sampling size studied by de Haan (2018) was smaller compared to Liberg, and that there were great variations in percentage FPT between the two farms included by de Haan. Depending on which factor that seems to contribute the most to failure of passive transfer, the etiology can be divided in three main categories. **Production failure**, which is essentially dependent on the quality of colostrum and its availability. **Ingestion failure**, when the quality of colostrum is adequate but the calf has been deprived from it during the critical time or otherwise unable to ingest it. When colostrum of good quality, within appropriate time and enough quantity has been administered to the calf, but despite that fails to achieve passive transfer, **absorption failure** is assumed (Tizard, 2013).

Illness is often associated with invasion of pathogens, which can be detected and neutralized in an early stage by maternal antibodies in calves with adequate passive transfer (Nonnecke *et al.*, 2012). In the event of challenge the calf must invest energy into mounting an immune response, energy that could otherwise be used for growth (Gelsinger & Heinrichs, 2017).

The gastrointestinal tract of neonatal animals produce little gastric acid or digestive enzymes. This protects immunoglobulin degradation, but also makes them more susceptible to bacterial contamination of colostrum (Person-Waller *et al.*, 2013; Baintner, 2007).

Colostrum

Components of colostrum

Definition of colostrum is different between authors (McGrath *et al.*, 2015). Persson-Waller *et al.* (2013) define colostrum as the first milking postpartum and this definition is used in this paper as well.

Colostrum differs from the secretions in the udder later during lactation. Although there are great variations in composition between species, this initial secretion generally contains more fat, protein, minerals and vitamins than regular milk (Sjaastad *et al.*, 2010). Colostrum is characterized by high content of immunoglobulins that transfers both into the blood of the neonate, contributing to systemic immunity and provide local protection in the gastrointestinal tract (McGrath *et al.*, 2015; Liberg 2000). IgG1 is the primary antibody in colostrum (the most abundant immunoglobulin-class 85-90% (Liberg, 2000)) and is derived from maternal serum (Weaver *et al.*, 2000; Heinrichs & Elizondo-Salazar, 2008). IgG is actively transported from blood of the dam to colostrum in the bovine species. Epithelial cells of the milk glands catch IgG from the blood by endocytosis and transfer it to colostrum via exocytosis. IgA and IgM are produced locally in the connective tissue between alveoli by plasma cells and play an important role in the local immunity of the mucus membrane of the neonate as well as the local defense in mammary epithelia against pathogens (Sjaastad *et al.*, 2010). Concentration of Ig in colostrum is highest directly after birth and will begin to decline if milking is delayed (Moore *et al.*, 2005). To get colostrum of optimal quality, producers should collect the colostrum within 1-2 h postpartum with a maximum delay of 6 h (Godden, 2008).

Besides the important nutritional value (Quigley & Drewry, 1998), and the role in passive transfer, colostrum contains a vast number of bioactive and growth- promoting substances (Heinrichs & Elizondo-Salazar, 2009). Lactoferrin, lysozyme, and lactoperoxidase have repressive effects on bacterial proliferation (Liberg, 2000). Colostrum influences the metabolism and stimulate development and function of the gastrointestinal tract (McGrath *et al.*, 2015). Compared to calves fed replacer (Roffler *et al.*, 2003) or colostrum deprived calves (Heinrichs & Elizondo-Salazar, 2009; Yang *et al.*, 2015), increased villous height, circumference, area, height/crypt depth ratio are seen for calves fed colostrum.

Colostrum quality

When talking about the quality of colostrum it usually refers to its content of immunoglobulin (Ig). A higher proportion of immunoglobulin in colostrum is regarded as better quality and the cut-off value of what colostrum is regarded to be of good quality is 50g Ig/L Ig (Liberg, 2000; Godden, 2008).

The concentration of IgG in colostrum has a great variation between individuals and values ranged from 4 to 174 g Ig/L of IgG in first milking colostrum in a group of Swedish dairy cattle. One fourth of these cows had a poor-quality colostrum, IgG < 50g/L (Liberg, 2000).

Factors affecting colostrum quality

Several factors have been shown to influence or are suspected to influence colostrum quality. The effect of time after birth, age of dam, breed and hygienic quality are some of the factors involved, although many more are suggested.

The concentration of Ig is affected by the time or number of milkings as IgG concentration declines rapidly postpartum (Godden 2008). Liberg (2000) recorded nearly halved concentrations of IgG in 2nd milking colostrum compared to 1st milking in average. Likewise, Moore *et al.* (2005) found rapidly declining concentrations of IgG as soon as 2 hours postpartum. Colostrum collected within 1-2 hours postpartum contains the highest amount of Ig. According to Godden (2008) first colostrum should be collected with a maximum delay of 6 h postpartum to milking.

A first parity cow does not necessarily have low concentrations of Ig in colostrum. Independent of age, a rather large variation in Ig content of colostrum can be observed between individuals (Liberg, 2000). This observation stresses the importance to test the quality of colostrum. However, studies have indicated a trend where cows of third lactation or above have higher Ig content than younger cows (Weaver *et al.*, 2000; Moore *et al.*, 2005; Godden 2008). de Haan (2018) recorded a similar pattern using Brix refractometer in quality testing of colostrum, showing significantly higher Brix values in colostrum from cows in third lactation or higher.

When addressing breed, statistically significant differences of colostrum IgG concentration between breeds have been established in several studies (Weaver *et al.*, 2000). In Sweden, the most common breeds of dairy cattle SRB and SLB/SH have been studied as well with different conclusions. Liberg (2000) compared colostrum quality between SRB and SLB but found no statistically significant difference between the two. de Haan (2018) could conclude within her material that SH cows had significantly higher IgG concentration in colostrum compared to Swedish red (SRB), based on Brix refractometer measurements.

Bacterial contamination of colostrum is another common problem of colostrum quality. TPC (total bacterial plate count) exceeding 100 000 colony-forming units (CFU)/ml, whereof >10 000 CFU/ml coliforms marks unacceptable contamination of colostrum (Persson-Waller *et al.*, 2013).

The colostrum bacterial count affect absorption negatively in calves. The mechanism to this observation is not determined but may be direct consumption of IgG in colostrum binding to bacteria and therefore becoming unavailable for absorption by the gut. Another suggestion is that bacteria interfere with the binding- site of IgG absorption (Persson-Waller *et al.*, 2013; Short *et al.*, 2016).

Elizondo-Salazar & Heinrichs (2009) found that calves fed heat-treated colostrum had a significantly higher IgG concentration in serum and greater AEA compared to calves fed raw colostrum at 24h of age.

Contamination at collection of colostrum is the predominant source of increased bacteria count in colostrum (Persson-Waller *et al.*, 2013). High bacterial count in colostrum results in decrease of passive transfer compared to colostrum containing less bacteria.

Factors affecting Ig absorption in the calf

AEA

Apparent efficiency of absorption (AEA) is a calculated value presented in percent, describing the efficiency of IgG absorbed into calf serum out of the mass IgG fed in colostrum to the calf. The measurement is calculated from the serum concentration of IgG in the calf times estimated or measured blood volume divided by colostrum volume and IgG concentration fed to the calf using the following formula: $AEA = ((\text{Serum [IgG]} * PV) / (\text{IgG intake})) * 100$, where $PV = 0.07 * \text{birth weight (kg)}$.

The values of AEA reported are highly variable between studies, but most of them are between 20% and 35% (Quigley & Drewry 1998; Osaka *et al.*, 2014). Halleran *et al.* (2017) calculated a broader range from 7.7% - 59.9% AEA in calves fed different volumes (minimum 4L fed) of colostrum. The group given 5.6 L colostrum instead of 4 L had generally higher AEA and higher total S-IgG (Halleran *et al.*, 2017). A few of the studies published measured the blood volume, others estimated. An estimate of 7% of body weight (BW) is commonly used.

Elizondo-Salazar & Heinrichs (2009) recorded a range of 16.3% - 28.5% in calves fed unheated colostrum and 19.7% - 34% for calves fed heat-treated colostrum. The hypothesis is that heat treatment may enhance AEA by reducing the number of microorganisms in colostrum and this would lead to less immunoglobulins being “consumed” by binding to and neutralizing pathogens in the colostrum

Great variation in AEA with an average of 41% was observed in Swedish calves by Liberg (2000).

Timing of ingestion

During the first 24-36h of life, the neonatal ruminant has a transient ability to absorb macromolecules such as immunoglobulins by enterocyte pinocytosis. This process is non-selective. Immunoglobulins are then transported to the bloodstream via the lymphatic duct (Weaver *et al.*, 2000).

At a certain time after birth, this route of absorption of macromolecules is terminated by a mechanism not fully understood. The event is called closure and the time to closure is variable between species and may be somewhat prolonged depending on feeding regime (Tizard, 2013; Weaver *et al.*, 2000). The closure is not abrupt. The capacity of absorption has been determined to peak during the first 4 hours of life and then start to taper (Weaver *et al.*, 2000). Osaka *et al.* (2014) found relatively modest reduction in AEA of colostrum IgG during the first 12 hours of life, followed by a more rapid decline in AEA. Feeding at different times within the first 4 hours of life revealed no significant difference in AEA according to Halleran *et al.*, (2017).

The results of Michanek *et al.* (1989), however, allow for some debate regarding the rate of reduction of intestinal permeability and age. Absorption of IgG from aliquoted colostrum was comparable between groups of calves fed their first meal colostrum at different ages up to 24 hours of age. During the sequent feedings, however, the group fed colostrum at 1 hour after birth had significantly higher absorption of marker-substance. These results give reason to believe that calves can transmit substantial amounts of macromolecules as late as 24 hours after birth when they are not fed colostrum previously.

Mass, method and volume of colostrum administered

FPT was reduced when calves received >100 g of IgG, and in the same study, few of the cows produced colostrum with an IgG concentration meeting this requirement in 2 L volume fed to calves (Weaver *et al.*, 2000). The only factor out of the ones studied by Nilsson (2015) that proved to have statistically significant differences between FPT and HPT (high passive transfer), was the mass of IgG (calculated) fed to the calves. Osaka *et al.* (2014) also concluded that AEA was significantly affected by the mass IgG consumed. Liberg (2000) found a correlation coefficient of 0.71 between mass IgG and serum concentration of IgG in the calves.

Slightly higher absorption of IgG has been demonstrated in calves fed by nipple bucket (this closes esophageal groove to abomasum) compared to esophageal tube, although the difference was not high enough to be statistically or clinically significant (Weaver *et al.*, 2000).

The higher rates of FPT observed in groups of calves allowed to suckle from the dam, is indicative of that dairy calves will not ingest adequate volumes without intervention to meet their immunoglobulin requirements (Weaver *et al.*, 2000).

Effect of dietary management

The dietary management of the dam during the dry period and her BCS at calving are other factors believed to affect passive transfer and general calf health. Transition from lactation to dry period is somewhat abrupt with great changes in diet. Management of dry cows and pregnant heifers is usually kept to the minimum. The drastic change in diet is aimed at reducing the risk of postpartum metabolic disorders, but may provide inadequate energy to the growing fetus during late pregnancy. If the diet of the dry cow is instead excessive, an increased BCS for the cow increases the risk for a more difficult calving and dystocia. This may lead to increased mortality in calves (Quigley & Drewry, 1998). Calves fed colostrum from a dam fed the restricted diet had a reduced absorption of Ig from colostrum compared to control (Quigley & Drewry, 1998).

Assessment of calf S-IgG concentration

Passive transfer in the calf can be evaluated by direct or indirect methods to determine if the concentration of Ig in the blood is adequate. Radial immunodiffusion (RID) is considered the golden standard where concentration of Ig is measured directly in colostrum or blood and is used in comparisons to other methods (Weaver *et al.*, 2000; Biemann *et al.*, 2010; Taylor *et al.*, 1996). However, the method requires laboratory settings, is relatively expensive and time-consuming. Another direct method to evaluate Ig concentration in blood or colostrum is quantitative ELISA (Weaver *et al.*, 2000). Gelsinger *et al.* (2015) compared performance between ELISA and RID, measuring Ig in unheated and heated colostrum and in bovine plasma. The ELISA is mentioned as a perhaps more economical alternative but is also performed at laboratory settings (Gelsinger *et al.*, 2015). The best correlation between RID and ELISA in this study was for bovine plasma, although the significant correlation was considered weak ($r=0.59$, $P<0.01$). ELISA was found to underestimate concentration of Ig compared to RID (predetermined criteria $CV<10.5\%$ variation between values).

Passive transfer in calves can also be assessed with indirect methods such as different refractometers (Deelen *et al.*, 2014; Weaver *et al.*, 2000; Taylor *et al.*, 1996). In this technique, serum or colostrum is evaluated for refraction to produce an estimation of total protein concentration. From this estimation of total protein, the Ig concentration can be calculated. Weaver *et al.* (2000) reported other studies to have found reasonable correlation ($r=0.72$) of refractometer readings and RID. McCracken *et al.* (2017) found a correlation of Brix refractometer to RID of $r=0.77$ and RID to TP refractometer $r=0.79$. Deelen *et al.* (2014) reported a high correlation of estimated STP by Brix refractometer to IgG concentration determined by RID, $r=0.93$. Refractometer tests have the advantage that they can be adapted for on-farm use and is easy and faster to perform. Wallace *et al.* (2006) compared two different refractometers measuring centrifuged and non-centrifuged serum samples with very high correlation ($r=0.96$) when estimating FPT in calves.

Calloway *et al.* (2002) compared different refractometers to RID as well as evaluate FPT classification and endpoints of the tests. The conclusion of these tests was that the refractometers performed similarly for classification of FPT. By moving the cutoff, the sensitivity and specificity of the tests changed. de Haan (2018) analyzed serum samples by optic and Brix refractometer with STP of $<55\text{g/L}$ as cutoff for FPT. Zakien *et al.* (2018) suggested a cutoff of $<52\text{g/L}$ STP for healthy calves and $<55\text{g/L}$ for dehydrated calves for classification of FPT.

To the author's knowledge, few studies have investigated the correlation between ELISA and refractometer in estimation of passive transfer. Zaiken *et al.* (2018) compared the performance of ELISA S-IgG concentration compared to digital Brix refractometer STP readings of calf serum and found an excellent correlation ($r=0.95$).

MATERIAL AND METHODS

Farms and study design

Samples and data used in this study were collected at two research dairy farms:

- 1) Lövsta SLU –The Swedish Livestock Research Centre
- 2) Röbbäcksdalen Research Station and Röbbäcksdalens Research dairy farm

From these two farms, in total 803 samples of calf serum were submitted to the study. Calves from Lövsta were Swedish Holstein, Swedish Red cattle (SRB) or cross breeds. All calves from Röbbäcksdalen were SRB.

Lövsta

During the study period (17th January 2015- 30th April 2017), 673 calves were born, from which 573 serum samples were available for analysis in this study. Calves were separated from the dam as soon as possible after birth. This routine was due to another study including the same calves. Calves were predominantly fed by nipple bottle, but calves that were too weak to suckle from nipple bottle were fed by esophageal tube. Blood samples from all calves were drawn at the age of two to seven days. Calves were given colostrum at the first meal without exception, but usually fed with transition milk from a later milking at the following meals. Calves born prior to 27th June 2016 were fed twice daily with colostrum/transition milk during the first four days. Calves born after 27th June 2016 were fed colostrum twice daily for three days.

Staff kept records of birth weight, breed, quantity and time of colostrum fed, origin of colostrum (if donor other than dam) and individual health records of the calves.

Röbbäcksdalen

Out of the 263 calves born from 24th February 2015 to 31st March 2017, 224 serum samples could be included in this study. At Röbbäcksdalen, newborn calves were transferred to individual pens shortly after birth. They were given first milking colostrum for three meals, fed twice per day. Then fed second milking colostrum for additionally four days. From age 5 to 10 days, calves were fed fresh, whole milk. Calves had ad libitum access to water, forage and concentrate during this period.

Quantity, timing and origin of colostrum (identity of donor if other than dams), as well as records of individual calf health were kept and submitted by the staff on the farm.

Management of colostrum samples

Sampling of first milking colostrum was carried out by staff at the farms. The time between birth and sampling was recorded, but the exact time for calving was not known in some cases. Samples were stored at -20°C until used for analysis.

Brix refractometer analysis of colostrum

Colostrum was analyzed with a Brix-refractometer (Digital hand held “pocket” refractometer PAL-1, ATAGOCO, LTD) to obtain an estimate for total protein content in a previous study (De Haan, 2018). Each sample was tested three times and the average value, visible contaminations or deviations and variation between tests were recorded. If there was more than 1 unit Brix % difference in value between tests, the sample was tested additionally 1-3 times by the same method.

In total, 604 colostrum samples from Lövsta and 230 samples from Röbbäcksdalen were analyzed with the Brix refractometer. Data from these measurements was available to be used in this study for calculation of AEA. Samples with visible deviations such as blood contamination, were not excluded from calculations and comparisons, but deviations were noted. Exclusively first milking samples were used.

Calculated IgG concentration of colostrum

The Brix results of the colostrum samples and identity of the dam were matched to the identity of the calf or calves that were given her colostrum. The estimated concentration of IgG was calculated according to the formula used by Quigley *et al.* (2013) $\text{IgG (g/L)} = -61.896 + 5.666 \times \text{Brix \%}$. This formula was used to simplify comparison of our results to similar studies. The total mass IgG was calculated based on information about concentration and volume of colostrum fed during the first 24 hours and where information was available, total mass IgG fed in the first meal. Information about the total mass IgG fed in the first meal was available for 267 calves at Lövsta, and AEA of those calves was used to test the correlation of the parameters.

Management of serum samples

Staff on farms collected blood samples from the calves at the age of 2-7 days. Samples were left to clot and transported to the biobank at SLU Uppsala, where they were centrifuged. The sera were kept stored deep frozen at -80°C until needed for ELISA test.

Serum samples analyzed by optic refractometer

Serum samples from 165 calves at Lövsta and 59 calves at Röbbäcksdalen were previously analyzed by optic refractometer to estimate STP (de Haan, 2018). Each sample was measured three times and the mean value calculated. The mean STP values of these calves were later used to compare with results from the ELISA assay in this study.

Enzyme linked immunosorbent assay

ELISA analysis

To obtain a more accurate value of Ig concentration in calf serum, a quantitative indirect ELISA was chosen for the analysis. A commercially available ELISA kit (Bethyl Laboratories, Montgomery, TX, USA) was used including: affinity purified sheep anti-bovine polyclonal coating antibodies, Horse radish peroxidase (HRP)-conjugated sheep anti-bovine polyclonal antibody and bovine reference serum. The procedure used was based on manufacturer's recommendations of the ELISA kit, but then tested and adapted to conditions at the lab to yield readings that could be measured by the equipment.

Table 1. Concentration of IgG and dilution factor for the standards used in the ELISA

Bovine reference serum (standard)	IgG concentration (ng/ml)	IgG concentration (mg/ml=g/L)	Dilution factor 1:2300 initial
1	500	0.0005	1:47000
2	250	0.00025	1:93000
3	125	0.000125	1:187 000
4	62.5	6.25e-5	1:373 000
5	31.25	3.125e-5	1:747 000
6	15.625	1.5625e-5	1:1 494 000
7	7.8	7.8e-6	1:2 988 000
8	0	0	0

Different dilutions of the coating antibody diluted in carbonate coating buffer were tried out in a 96-well microtiter plate. Bovine reference serum was diluted according to the manufacturer's recommendation to make a standard curve (see table 1). The concentration of HRP-conjugated sheep anti-bovine antibodies had to be tested in several dilutions and with different incubation times.

The efforts resulted in the following procedure:

1. Dilutions of calf serum were prepared in a six step dilution series in clear 96-well plates and stored at -20°C until analysis.
2. 96-well ELISA plates were coated with 100 µl of affinity-purified polyclonal sheep anti-bovine IgG in carbonate coating buffer (1:250 dilution), and left to incubate at 4° C overnight
3. Residual, unbound coating-IgG was washed away with phosphate buffered saline with 0.05% Tween 20 (PBS-T) as washing solution in a five step wash
4. Blocking of unspecific binding sites with (PBS-T) and incubation in room temperature for 30 min

5. Serum samples were added at dilution 1: 112 000 initially. Samples with IgG concentration above or below our standard reference curve with these settings were retested in appropriately adjusted dilutions (1:224 000 or 1:16 000)
6. 100µl of samples and standards were added in duplicate to wells and incubated in room temperature for 90 min. Then samples were removed and wells washed five times using PBS-T before 100 µl HRP-conjugated sheep anti-bovine IgG (1:75 000) was added to the wells and incubated for 60 min in room temperature. HRP-conjugate was removed and wells washed five times with PBS-T
7. TMB HRP-substrate solution was then added to each well and left in the dark for 15 min for color to develop. Stop solution (0.4 M sulfuric acid) interrupted the reaction and absorbance at 450 nm was read immediately

Variation of ELISA analysis

Variation was calculated to determine if precision of the method within plate (intraplate) and between plates (inter-plate) was within reasonable limits. Intraplate variation (i.e., variation between duplicates on the same plate), inter-plate variation during the same day and inter-plate variation between different days was calculated. The absorbance values from the most concentrated standard (ST1) was used for the inter-plate comparisons. Maximum variation between plates measured the same day was calculated using the average absorbance with greatest difference between plates, thus obtaining a maximal inter-plate variation that day. To get inter-plate variation between days, the average absorbance from the plates with greatest difference in mean absorbance that day was used and compared to the corresponding average absorbance data from plates analyzed the next day.

S-IgG results exclusion criteria

In total 803 calf serum samples were analyzed to quantify concentration of IgG, and valid results were obtained from 700. Samples retested due to values outside the range of the ELISA standards or with too high variation between duplicates (>20%) were retested once, twice or even a third time. Extremely high results of serum samples with the ELISA were excluded according to different criteria due to if they were retested or not. Extreme values of samples tested only once were excluded if they measured above 95 g/L, because values this high were very unlikely to be correct according to literature (Gelsing & Heinrichs, 2017; Short *et al.*, 2016; Morrill *et al.*, 2013; Deelen *et al.*, 2014). Most of the retested samples had a high variability, and were completely excluded if the variation exceeded 20% between measurements. Intra-plate variability was also taken into consideration. Measurements with variability >20% between duplicates or when only one of the duplicates was measurable, were excluded from further comparisons. Valid values were used for calculations and comparisons, and where samples had more than one acceptable reading, mean values were used.

Calculation of AEA

Intake of IgG, serum IgG and AEA of IgG were calculated according to Quigley and Drewry (1998), where $AEA (\%) = [\text{serum IgG (g)}/\text{IgG intake (g)}] * 100$.

$\text{IgG intake (g)} = \text{IgG concentration in milk (g/L)} * \text{milk volume (L)}$.

$\text{Serum IgG (g)} = \text{IgG concentration in serum (g/L)} * (\text{Body weight (BW)} * 0,07)$

Statistical analysis

Minitab express version 1.3.0 was used for statistical analysis of the data. Descriptive statistics of S-IgG and AEA with number of samples, mean, median, standard deviation, minimal, and maximal values were obtained for each farm separately and combined. The graphs (histogram, individual value plot, boxplot, scatterplot) were also created with Minitab express.

A 2-sample t-test in Minitab express was used to compare the mean S-IgG of unhealthy and healthy calves and AEA of healthy and unhealthy calves both including only calves from Lövsta.

Spearman correlation was also performed in Minitab express and used to evaluate correlation between results of S-IgG (analyzed by ELISA) and refractive index (using optic refractometer). Spearman correlation was used to calculate linear correlation between AEA and calculated mass IgG fed in first meal (this comparison including only 267 calves from Lövsta). Pearson correlation test in Minitab express was used to calculate linear correlation between AEA and birth weight.

A simple regression between refractive index obtained by optic refractometer and S-IgG values from ELISA, was used to describe the linear relationship found between measurement of optic refraction of calf serum to the concentration of IgG in serum. The regression is illustrated by a graph with an equation.

RESULTS

Serum concentration of IgG and AEA

Valid S-IgG results from the ELISA are shown in table 2 and distribution of AEA is shown in table 3. AEA values above 100% were excluded since it is not possible for calves to absorb a larger amount of IgG than they have been fed.

Table 2. Distribution of values for S-IgG for calves age 2-7 days

	Lövsta n=503	Röbäcksdalen n=197
Range of S-IgG (g/L)	1.42 –91.60	1.11 –71.97
Mean +/- SD (g/L)	25.06 +/- 13.64	16.00 +/- 10.25
Median (g/L)	23.00	14.40
S-IgG <10g/L	48 (9.5%)	60 (30.4%)

Table 3. Distribution of calculated AEA the first 24 hours postpartum

	Lövsta (n=366)	Röbäcksdalen (n=72)
Range of AEA (%)	2.74- 96.63	2.03- 91.61
Mean +/- SD (%)	18.14 +/- 12.98	19.67 +/-18.46
Median (%)	14.44	13.03

Correlation between ELISA and optic refractometer (STP)

Serum samples from 165 calves at Lövsta and 59 calves at Röbäcksdalen, were previously analyzed by optic refractometer to estimate STP (de Haan, 2018). The same serum samples were measured in the IgG-ELISA. Simple regression between values from both methods is presented in figure 1. Equation of regression between S-IgG and refractive index was following: ELISA IgG (g/L) = $-57,527 + 13,2837$ Mean serum %optic ref.

Correlation between optic refractive index and ELISA S-IgG was 0.75 ($p < 0.0001$, Spearman's rank correlation coefficient) when including calves of both farms.

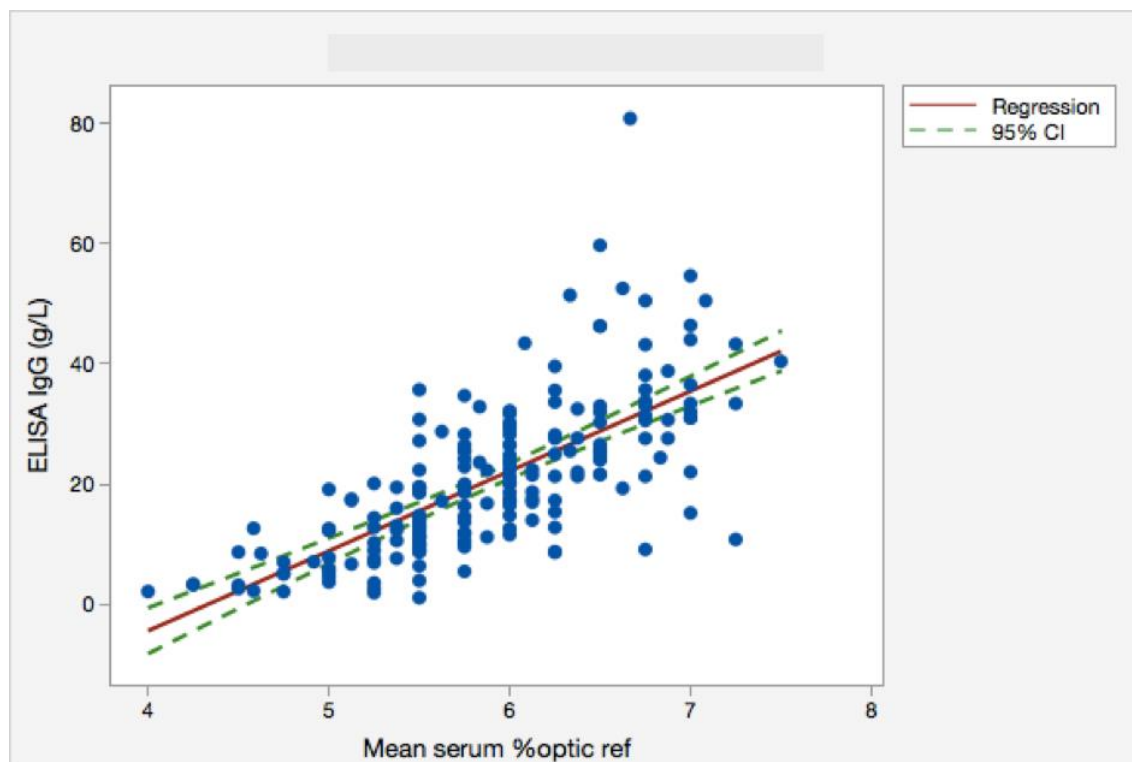


Figure 1. Scatterplot illustrating the regression between samples analyzed by optic refractometer and ELISA.

Proportion of FPT calves based on IgG vs STP

There were 44 calves at Röbbäcksdalen analyzed for STP and S-IgG (table 4). Assessment of STP categorized 21 of them (47%) as FPT given their serum having less than 55g/L. Only 15 (34%) of those calves were classified as FPT according to S-IgG measurement (cut-off <10 g/L).

Table 4. Healthy and ill calves from Röbbäcksdalen grouped according to different criteria of FPT

Röbbäcksdalen N=44 Cut-off in g/L	Number (proportion)	Registered health remarks	Calves perceived as healthy
STP < 55	21 (47%)	9	12
STP > 55	23 (53%)	5	18
S-IgG < 10	15 (34%)	7	8
S-IgG < 15	23 (53%)	9	14
S-IgG > 15	21 (47%)	5	16

The number of calves at Lövsta that had their serum tested by both methods were 153. They were also compared regarding number of FPT cases classified by each method (table 5).

Table 5. Calves from Lövsta grouped according to different criteria of FPT.

Lövsta N=153	Number (proportion)	Registered health remarks	Calves perceived as healthy
STP < 55 g	32 (22%)	6	26
STP>55 g	121 (78%)	28	93
S-IgG <10 g	25 (16%)	6	19
S-IgG<15 g	54 (35%)	13	41
S-IgG>15 g	99 (65%)	21	78

Variation in percentage of measurements of optical density in the most concentrated standard of each ELISA plate is presented in table 6 below.

Table 6. Inter-plate and intra-plate variation of ELISA optical density values

	Day 1	Day 2	Day 3	Day 4	Day 5
Mean intra-plate	1.1%	2.7%	1.2%	2.0%	1.8%
Max intra-plate	1.8%	3.2%	2.9%	3.2%	3.3%
Min intra-plate	0.1%	2.2%	0.1%	0.3%	0.3%
Max inter-plate that day	5.1%	3.9%	5.9%	10.7%	2.4%
Inter-plate variation between days	Day 1- day 3 5.3%	Day 1-day 2 8.5%	Day 2-day3 3.2%	Day 3-day 4 2.8%	Day 4-day 5 8.9%

AEA of calves with S-IgG <10g/L

Only 55 of the calves which classified as having FPT could get an estimate for AEA. The reason for this was that those calves (<10 g/L S-IgG) had missing data on birthweight, brix- value or volume of the colostrum fed and were therefore excluded in this comparison. Distribution of AEA in calves with FPT separated by farm is presented below in table 7 and as a boxplot in figure 4. Mean AEA of calves classified as having FPT within each farm was compared with mean AEA of all calves in that farm and tested with a 2-sample t-test. The mean calculated AEA of calves with FPT was found to be significantly lower compared to the average within each farm (figures 3 and 4).

Table 7. Distribution of AEA among calves with FPT

	Lövsta n=31	Röbäcksdalen n=24
Range of AEA (%)	2.7 –27.1	2.0 –54.3
Mean +/- SD (%)	8.0 +/- 5.8	10.3 +/- 2.6
Median (%)	6.6	7.3

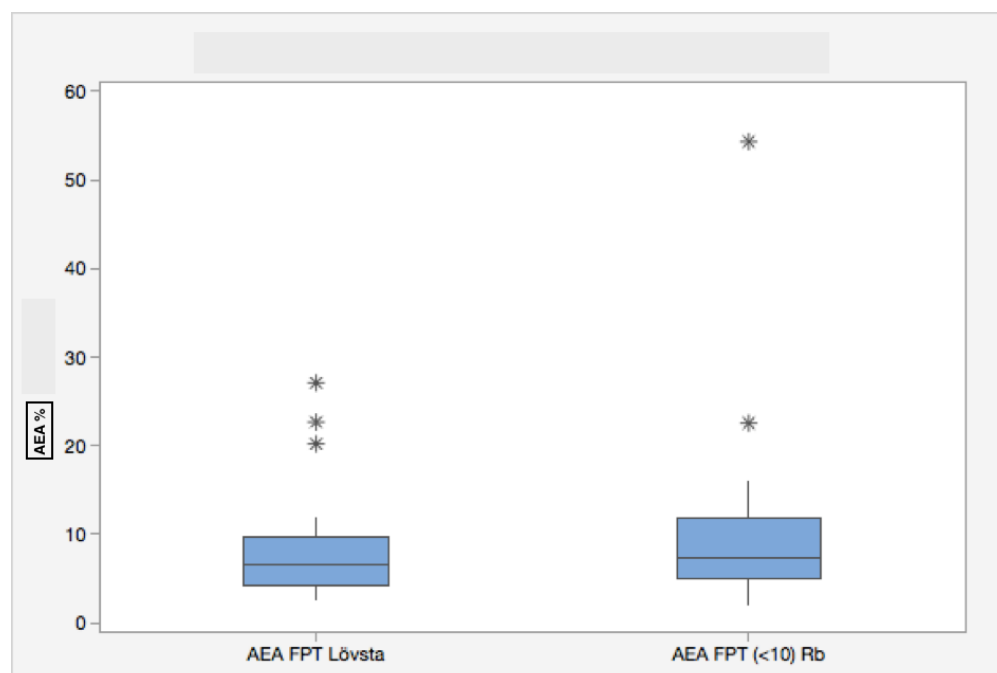


Figure 2. AEA distribution of FPT (<10g/L) cases. Lövsta and Röbäcksdalen.

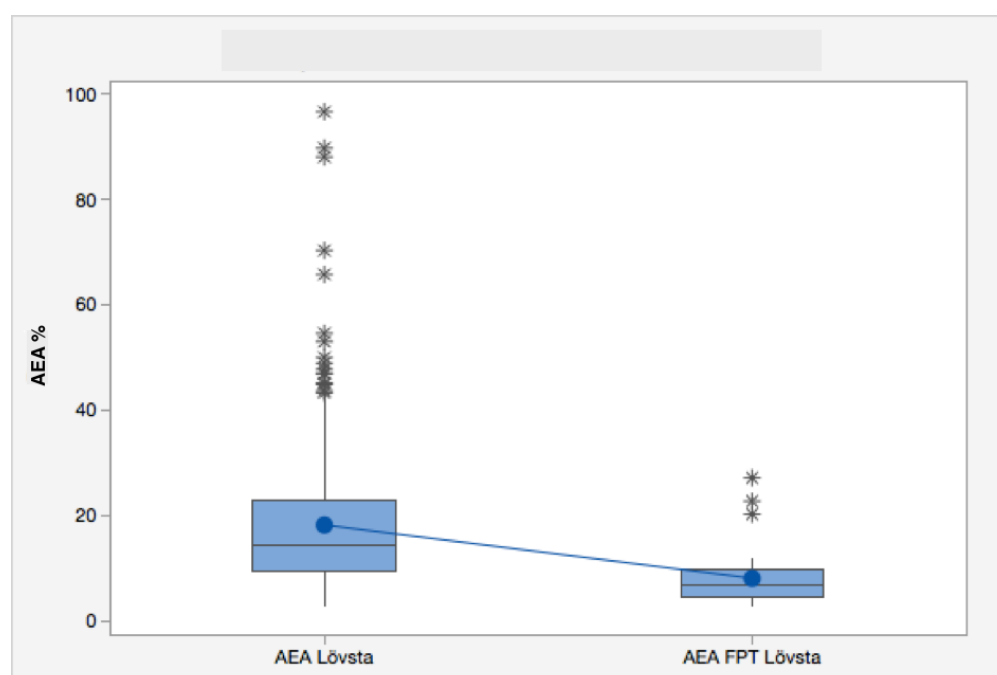


Figure 3. AEA of all the calves from Lövsta compared to AEA of calves classified as FPT. Mean AEA was significantly lower in calves with FPT (2-sample t-test $p<0.001$) compared to the mean AEA of all calves from Lövsta.

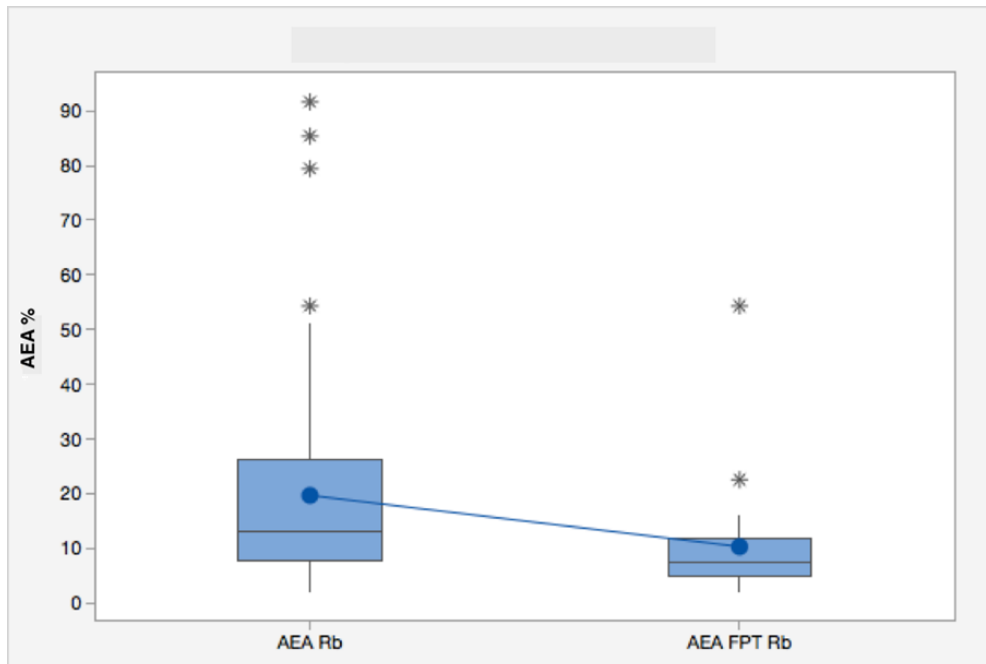


Figure 4. AEA of all the calves from Röbbäcksdalen Mean AEA was significantly lower in calves with FPT (2-sample t-test, $p=0.003$ for Röbbäcksdalen) compared to the mean AEA of all calves from Röbbäcksdalen.

AEA and birth weight

Distribution of birth weight and AEA from 365 calves from Lövsta is presented in a scatterplot with regression line (figure 5), with values of AEA above 100% excluded. No significant linear correlation between AEA and birth weight was found with Pearson correlation test $r=0.09$, $p=0.08$. The average birth weight from this group of calves were 41.8 ± 5.86 kg. Lowest weight recorded among the calves with AEA calculation was 25.8 kg while the maximum weight was 71 kg.

When calculating the entire weight data from calves born during the project, the mean birth weight was 41.7 kg (range 22-71 kg).

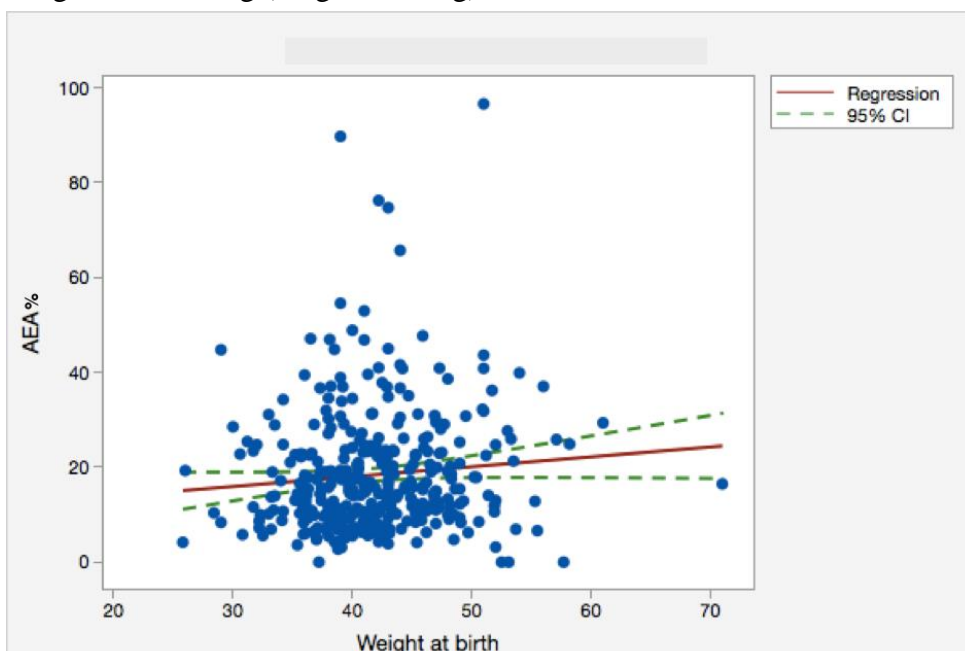


Figure 5. Scatterplot and regression analysis of AEA in relation to birth weight of 365 calves from Lövsta.

At Lövsta, 138 calves had registered signs of illness and the most common remark was diarrhea. Calves were placed in groups according to presence of registered illness or not and S-IgG and AEA were compared between the groups (figure 6). Among unhealthy calves, 117 had a valid value of S-IgG. Unhealthy calves had a significantly higher mean S-IgG (27.4 g/L) than calves perceived as healthy (24.3 g/L) (2-sample t-test, $p=0.04$).

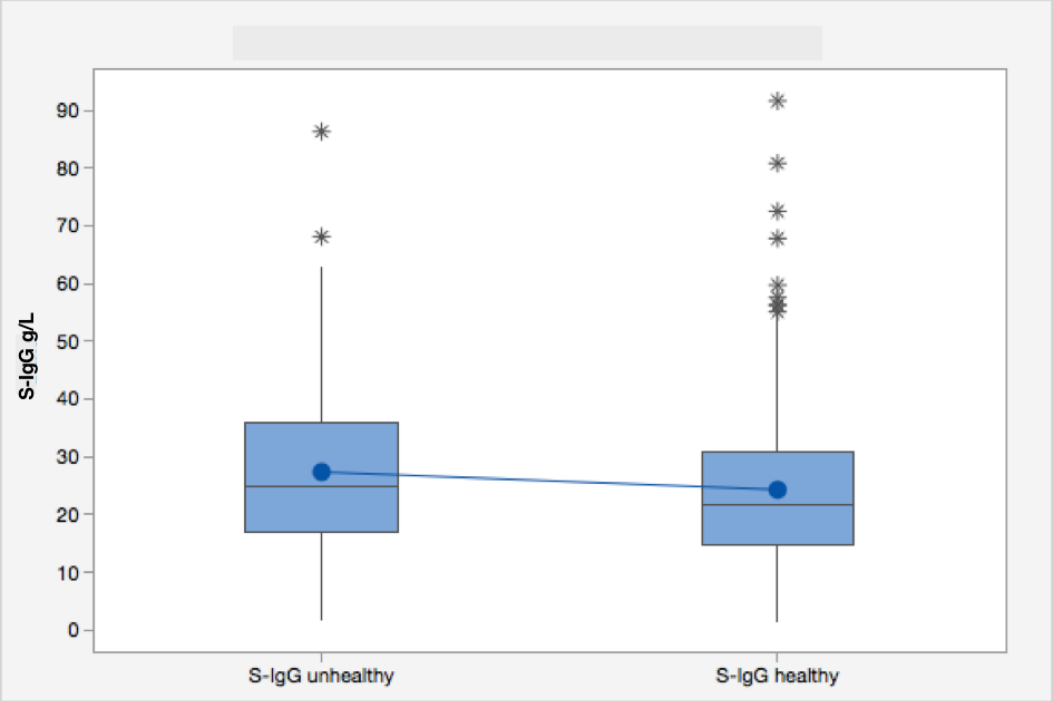


Figure 6. Boxplot of S-IgG for calves either healthy or unhealthy. Lövsta. The group of healthy calves had a 3g/L lower mean S-IgG concentration ($p=0.04$) than the group of calves with registered illness 2-sample t-test.

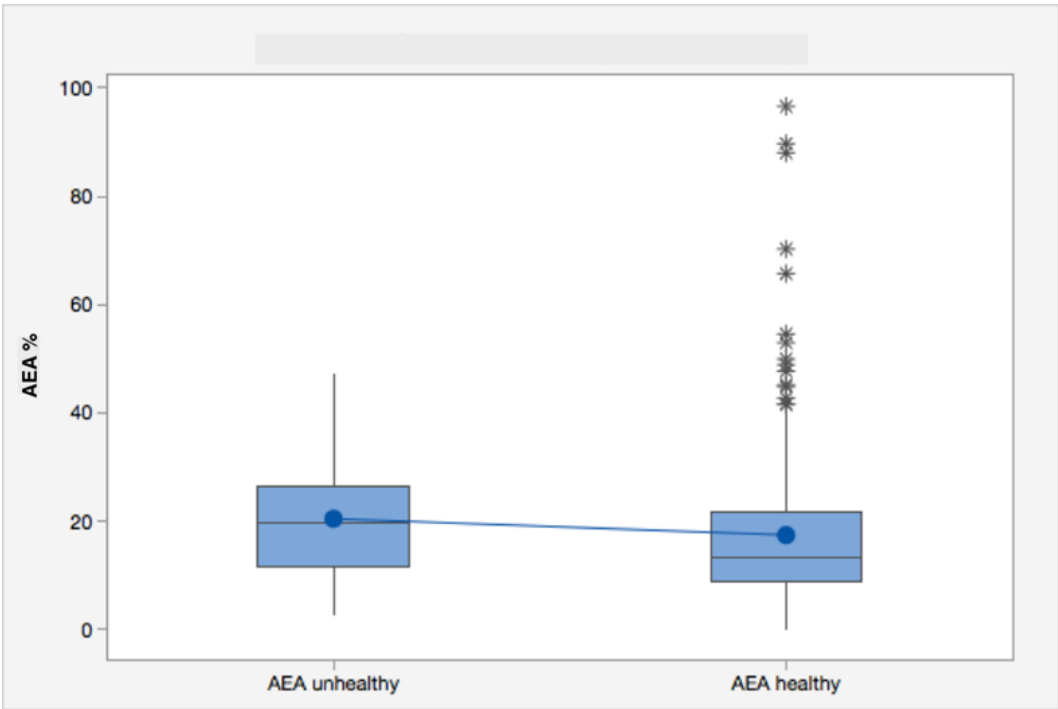


Figure 7. Boxplot of AEA from ill and healthy calves from Lövsta

AEA could be estimated in 78 of unhealthy calves at Lövsta (figure 7). The AEA of all these calves is illustrated by the left box in the plot. On the right are the calves perceived as healthy (n=287). The mean AEA of unhealthy calves was 20.44% +/- 10.28, and of healthy calves 17.42% +/-13.51 (3 percentages lower, 2-sample t-test (p=0.03).) (n=287). The mean AEA of unhealthy calves was 20.44% +/- 10.28, and of healthy calves 17.42% +/-13.51 (3 percentages lower, 2-sample t-test (p=0.03).)

The effect of total IgG content in the first meal on AEA

No significant correlation between total mass IgG in the first meal fed, and AEA, was found with Spearman correlation test $r=0.07$, $p=0.26$ and the data is shown in a scatterplot (figure 8). The calves were all from Lövsta (n=210). Most of the calves had ingested >100 g IgG in their first meal with a mean of 215.6 g (range 19.2-549.6).

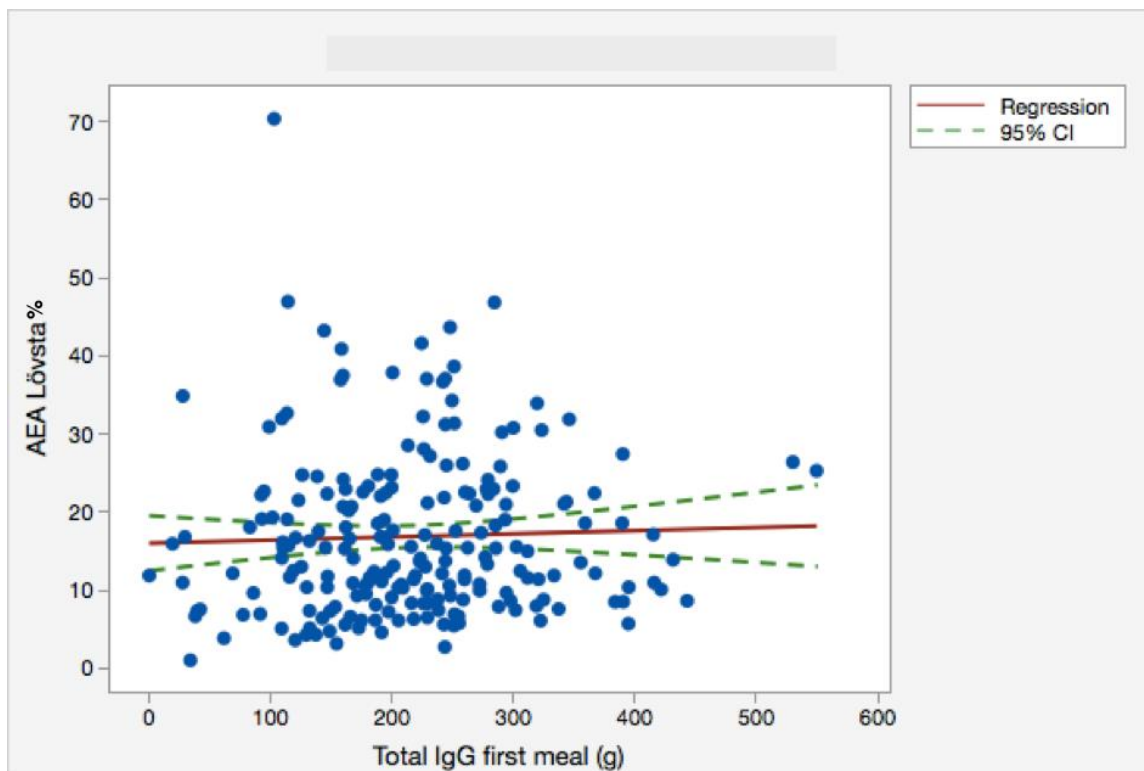


Figure 8. Scatterplot and regression analysis of AEA compared to mass IgG given in first meal, Lövsta.

DISCUSSION

Serum IgG compared to STP and proportion FPT

This study recorded data of almost all calves born during the study period at two farms, giving a very detailed view of the colostrum quality of the cows and the characteristics of the calves as well as routines for colostrum feeding but only of these two farms. Differences in breed and routines in colostrum feeding between farms may be important in this comparison. The proportion of calves classified as FPT was different when measurements were made with S-IgG ELISA compared to estimations of STP with refractometer (de Haan, 2018). Although results of samples analyzed by both methods corresponded fairly well (Spearman's rank correlation test $r=0.75$). S-IgG measured by ELISA likely tended to overestimate the level of passive transfer compared to Brix and optic refractometer. Morrill *et al.* (2013) presented better correlation ($r=0.86$) between calf serum refractive index (determined by Brix refractometer) and S-IgG (determined by RID). Zakian *et al.* (2018) also showed better correlation, $r=0.95$, between values determined by ELISA and digital Brix refractometer. Unfortunately, Zakian *et al.* (2018) had no description of their successful ELISA protocol in detail. The suspected overestimation of S-IgG in the ELISA is perhaps one of the reasons that the two methods classify the calves so differently.

In both studies, the calves were sampled at between 2-7 days of age. Calf serum concentration of Ig originating from colostrum is expected to decrease over time, and half-life of 17.9 days (Besser *et al.*, 1988) up to 28.5 days (Murphy *et al.*, 2014) has been reported. Wilm *et al.* (2018), also followed changes in serum Ig concentration during the first 10 days of life. Results indicated very good correlation (Pearson $r \geq 0.88$) in measurements of S-IgG concentration between 24 h and up to 9 days of age in a group of Holstein calves. S-IgG was observed to decline at a rate of approximately 0.69 ± 0.05 g/L per day. Based on these observations, sampling delayed for 7 days (for some of the calves) might have influenced the result by causing a decrease in S-IgG below the cut-off for FPT at 10g/L, hence falsely classifying a few of the calves as cases of FPT. However, this is only likely to have happened to calves with S-IgG close to the cut-off and in cases where sampling was delayed. This is expected to have happened in a few cases and certainly does not explain the unexpectedly high Ig concentrations found with analysis by ELISA.

Dehydration of the calf can influence the concentration of S-IgG through hemoconcentration and is therefore taken into consideration by several other studies of passive transfer in calves. This factor was not taken into consideration or compensated for in this study, which could have given some of the samples a false high value compared to animals in normal hydration status.

Passive transfer and health

When comparing the results to the data of illnesses for the calves of this period, the cut-off for FPT at 10g/L seems too low since many calves with PT above 10g/L had registered illness. The cutoff value for acceptable PT of 10g/L Ig was established by RID, not ELISA (Tyler *et al.*, 1996; Furman-Fratczak *et al.*, 2011) and the methods are not directly comparable (Gelsinger *et al.*, 2015). Overestimation of S-IgG is probably involved in falsely classifying some of the calves with S-IgG >10g/L to have acceptable PT in this study. In the study by Furman-Fratczak

et al. (2011), there was evidence that serum concentration IgG >10 g/L was enough to protect the calves from falling ill before 14 days of age, while calves included in my material was still largely susceptible to illnesses when serum Ig exceeded this benchmark when looking at the number of calves affected. A larger proportion of calves remained visibly healthy when concentration IgG was ≥ 15 g/L. Other studies also indicate that Ig of 10g/L is too low to be regarded as sufficient PT: Furman-Fratczak *et al.* (2011) reported a better protection against respiratory disease in calves with ≥ 15 g /L S-IgG. Virtala *et al.* (1998) studied risk factors of pneumonia among calves and found that S-IgG of >12g/L had a stronger protective effect than S-IgG >8g/L.

Confusingly, there was a statistically significant lower mean S-IgG in a group of apparently healthy calves at Lövsta compared to the group of calves with recorded illness. The significance of this result is unclear and mean S-IgG of the both groups were both well above the cutoff for FPT at <10g/L. The results of lower S-IgG in healthy calves agrees with the previous results by de Haan (2018). She presented a statistically significant higher STP in calves from Lövsta with longer duration or more severe illness than in healthy calves. These calves were more likely to suffer from dehydration. No statistically significant difference was seen in her study between STP of healthy calves compared to calves with mild-moderate illness and duration or in total (not differentiating severity of illness) between healthy/ill, but IgG tended to be lower for healthy calves.

In the previous work by de Haan, there was a significantly higher STP in untreated calves compared to calves given treatment at Röbbäcksdalen. Unfortunately, there was not enough data from younger calves included in this study to repeat the comparison for Röbbäcksdalen.

Calves in this study were fed transition milk for an additional 2-3 days, and while IgG content is not expected to contribute to the serum concentration past gut closure, but it might provide local protection. Conneely *et al.* (2014) reported lower odds for signs of illness in calves that were fed transition milk with high IgG content (63.3 g/L) after their first meal colostrum compared to calves that received no subsequent feedings of colostrum or transition milk after their first meal. There are however more differences between the groups included in this study that may be of importance for their level of passive transfer.

Results AEA

Despite the difference in mean S-IgG levels between farms, AEA from calves were remarkably similar in range, mean and median. Impossibly high values above 100% were calculated for a few animals and excluded from comparison. Extreme values suggest that some of the data might be incorrect. These calves had probably obtained more colostrum than was declared in the records (calves born during the night that had opportunity to suckle), but other sources such as over-estimation of concentration of S-IgG by the ELISA may have contributed to the extremely high AEA. Calves weighed at different ages contribute to the source of error in calculation of AEA. The maximum estimations of AEA in this material above 90% also seems suspiciously high, but very high values are also reported by Lago *et al.* (2018) with 76.9% in the colostrum fed group. As mentioned previously, other studies have calculated a narrower range of AEA usually between 7-59% (Quigley & Drewry, 1998; Osaka *et al.*, 2014; Halleran *et al.*, 2016;

Liberg, 2000). In my calculations of AEA, 7% of BW was used in the calculation to estimate plasma volume as per Quigley & Drewry (1998), while other studies have estimated a higher factor for % plasma per kg BW. This might have led to underestimation of AEA in this study compared to the studies using an estimate for plasma volume greater than 7%.

Birth weight or mass IgG fed at first meal did not significantly affect AEA in this study, while as mentioned earlier Liberg (2000), Nilsson (2015) and Osaka *et al.* (2014) all found that AEA was positively correlated to mass IgG fed. There are several limitations in this study such as the choice of correlation analysis that is best suited for linear associations. It can only be concluded here that no linear correlations were seen between these parameters. It is possible that non-linear associations exist, but these are not detected by these statistical methods. Again, some of the calves were weighed later than the first day of life and this may also have affected the calculations slightly.

AEA of ill and healthy calves

Health data from calves from Lövsta alone were included in the comparison. Röbbäcksdalen had too few estimates of AEA and many incomplete records and therefore had to be excluded in some comparisons. The calves at Lövsta without remarks on health had surprisingly a lower mean AEA than the group with registered illness. My expectation was that calves with lower AEA would prove more prone to develop health issues at least since they would get a lower S-IgG concentration than a calf with more effective uptake. As shown in figure 6 and 7, AEA, like S-IgG was lower in the healthy group of calves than in the unhealthy.

AEA in calves with FPT

Since the concentration of IgG in serum is used in the calculation for AEA, interpreting results should be done cautiously. Mean AEA was significantly lower in calves with FPT (classified as S-IgG <10g/L) in each farm (2-sample t-test $p < 0.001$ for Lövsta and $p = 0.003$ for Röbbäcksdalen) compared to the mean AEA of all calves at the respective farm (figure 3 and 4). This result needs to be supported by other studies before any clear conclusions are made, but it seems that there are indications of inefficient absorption in at least some of the calves with FPT.

Establishment of IgG ELISA

Establishment of a functional ELISA protocol became a significant task of this paper. The dilution and incubation time of main constituents of the ELISA, i.e. coating antibody, conjugated antibody, samples and reference serum were systematically evaluated.

Coating antibody at dilution of 1:250 proved to be the most useful as it gave comparable results to 1:100 dilution. Coating antibody was consistently incubated at +4°C overnight to be used the following day. Background signal produced by distilled water and PBS-T was low during the initial testing and remained at a satisfactory level throughout the study except for two separate occasions. The generally low background signal made the dilution buffer a sufficient choice as blocking agent for unspecific binding sites after coating, and additional blocking agents such as horse serum or fish gelatin were never used.

Initially, the conjugated antibody was diluted at 1:50 000 and 1:100 000 with 60 min incubation time at room temperature. Dilution at 1:100 000 gave a faint signal, while 1:50 000 produced a slightly too strong signal. To get better readings, a concentration of 1:75 000 with 90 min incubation time was tested. The prolonged incubation time of HRP conjugated antibody resulted in “over-exposure” increasing the noise and variation between duplicates. However, a dilution of 1:75 000 combined with an incubation time of 60 min gave greatly improved results with better consistency and was therefore selected in the final protocol.

Dilution series

Serum samples were diluted in PBS-T and different dilution series were tested. Initial attempts starting at 1:20 000 gave too concentrated samples and the dilution was gradually increased. Diluting very small volumes of sample into relatively large volumes of dilution buffer (fewer steps to desired dilution) produced highly variable values between measurements of the same samples. Much time and effort was spent to test several dilution series with the aim to create a more consistent result. In the final protocol, serum samples were diluted in clear well plates in six steps, diluting the sample 6.94 times in each step (36µl sample into 214µl dilution buffer) reaching 1:112000 dilution in the final step. With every step in the dilution series there is a risk of introducing error. The large number of samples necessitated the work to be divided into steps, when ideally all samples should be prepared during the same circumstances to minimize error. A dilution of similar strength, (1: 100 000) as the one used in our protocol, was used by Gelsinger & Heinrichs (2017) in determination of Ig concentration in calf serum and plasma. Gelsinger *et al.* (2015) initially used a dilution factor of 50 000 when determining IgG concentration of bovine plasma by ELISA. In a second, smaller comparison of ELISA and RID, calf plasma samples 48h after birth were used in a dilution of 1:500 000 (Gelsinger *et al.*, 2015). According to these authors, the ELISA was underestimating the IgG concentration compared to the golden standard test RID. Gelsinger *et al.* (2015) experienced that a greater proportion of samples needed to be retested with the ELISA. RID was not used for comparison during the work with ELISA in the current study, but we also experienced that samples tested with ELISA had to be re-measured in many cases. It was assumed due to trouble with the dilutions. Thus, RID is likely a more consistent method to measure Ig concentration (Gelsinger *et al.*, 2015). In the present study, ELISA was likely overestimating the IgG concentration due to the very high results obtained.

During calibrating tests of the ELISA, some variation in absorbance of the standard became obvious and it was not always within the optimal range for the absorbance of plate reader. Therefore, reference standard dilutions for the entire sample run were made in greater volume, vigorously vortexed, aliquoted (1.2 ml) in cryotubes and stored at -20°C until being used, as an attempt to further standardize the conditions of the ELISA. One set of standards were thawed and tested in an ELISA prior to analyzing samples to ensure that readings were within optimal limits of the equipment. Not more than two freeze-thaw cycles per aliquoted standards were allowed.

Interpretation of results and issues with ELISA

Considerable effort was made to optimize the ELISA protocol, but despite this, a few samples demonstrated deviating results. Samples reaching results >70g/L S-IgG were observed in six

samples from Lövsta and four from Röbbäcksdalen. Based on reports from other studies of normal range of S-IgG, results of S-IgG above 70g/L would be considered extreme values (Halleran *et al.*, 2017; Deelen *et al.*, 2014; Elizondo-Salazar & Heinrichs, 2009; Zakian *et al.*, 2018). In the previous work by de Haan (2018), the maximal level of STP from Lövsta, Röbbäcksdalen and Viken was 80g/L, indicating that the ELISA assay is overestimating S-IgG levels compared to measurements by optic refractometer. However, correlation of results with samples measured by both these methods was relatively good ($r=0.75$ Spearman correlation test), indicating a linear relationship between the two methods. McCracken *et al.* (2017) measured STP with a calculated range of S-IgG between 2.3 to 65.5 g/L. Gelsing & Heinrichs (2017) reported S-IgG of 20.4 g/L in the group fed unheated colostrum and 21.7 g/L among the calves fed heat treated colostrum. Extreme values of S-IgG would need to be validated with new ELISA readings in freshly diluted samples at the very least, and preferably controlled also in other dilutions or with additional methods (RID or refractometry) to check accuracy in the values obtained. Values from repeated tests were included in a calculated mean if the variation between results was within reasonable limits ($<20\%$). In some cases, the only value obtained was an extreme value and it would be interesting to repeat those tests. Unfortunately, there was no more time to do so and it was not the main objective in this paper to investigate the method more thoroughly. Repeated problems in analysis of a few of the samples could be due to pre-analytical or analytical errors. Samples with lower concentration IgG seemed to show better consistency when tested repeatedly.

CONCLUSION

S-IgG determined by ELISA in this study tended to overestimate the values. When comparing result of S-IgG to refractive index analysed by optic refractometer, it was found to have a lower correlation than similar comparisons in the recent studies. Health-registration indicated that minimum limit for acceptable PT of $>10\text{g/L}$ was likely too low and should be raised when using this ELISA, based on comparison of number of FPT cases detected by optic refractometer.

Mean AEA was significantly lower in calves with FPT in each farm (2-sample t-test $p<0.001$ for Lövsta and $p=0.003$ for Röbbäcksdalen) compared to the mean AEA of all calves from Lövsta and Röbbäcksdalen. Since AEA is calculated partially from S-IgG, these results should be interpreted with caution, but suggests that low AEA might affect rate of FPT. Average AEA and S-IgG was surprisingly higher in the group of calves with registered illnesses than healthy calves, and from this result low AEA seems to have little negative impact on general health if S-IgG concentration is acceptable.

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