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ADDITIONAL UNPUBLISHED WORK

EVALUATION OF DIFFERENT ANALYTICAL METHODS TO ASSESS FAILURE OF PASSIVE TRANSFER IN NEONATAL CALVES

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ABBREVIATIONS

AEA	apparent efficiency of absorption
a.m.	morning
AUC	area under the curve
BW	body weight
bzw.	beziehungsweise
CA	presence of the calf group
CE	capillary electrophoresis
CGE	capillary gel electrophoresis
CI	confidence interval
CON	control group
°C	Celsius
d	day(s)
d.h.	das heißt
dL	deciliter
DIM	days in milk
e.g.	exempli gratia
ELISA	enzyme-linked immunosorbent assay
et al.	et alii/aliae/alia
FPT	failure of passive transfer
g	gram(s)
g	gravity
h	hour(s)
H_2O_2	hydrogen peroxide
ID	identifier
i.e.	id est
IE	internationale Einheit
Ig	immunoglobulin
i.m.	intramuscularly
IU	international unit
kg	kilogram(s)
kPa	kilopascal
L	liter
min	minute(s)
M	molar
m	meter(s)
mg	milligram(s)
mL	milliliter
mm	millimeter

MP	metabolizable protein
n	number of samples
NAHMS	National Animal Health Monitoring System
nm	nanometer
NPV	negative predictive value
OXY	oxytocin group
P	probability
PBS	phosphate-buffered saline
p.m.	afternoon
PPV	positive predictive value
r	Pearson correlation coefficient
R^2	coefficient of determination
RID	radial immunodiffusion
ROC	receiver operating characteristic
s	second(s)
SD	standard deviation
SE	standard error
Se	sensitivity
Sp	specificity
TIA	turbidimetric immunoassay
TMB	tetramethyl-benzidine
TMR	total mixed ration
TP	total protein
TS	total solids
UV	ultraviolet
µg	microgram(s)
µL	microliter
vs.	versus
w	half-width(s)
wk	week(s

1 INTRODUCTION

Calf mortality still represents a major welfare issue and economical problem in the modern dairy industry. Calves are exposed to several hazards immediately after birth within the first 2 – 5 weeks of life (Mee et al., 2008; Mee et al., 2013). These weeks are considered a high-risk period, especially as the calf's ability to initiate an immune response is insufficient during this period. Calves are born being agammaglobulinemic and therefore immunonaive (Weaver et al., 2000; Barrington and Parish, 2001; Mee et al., 2013). The transfer of immunoglobulins in utero to the fetus is not possible, due to the impermeability of the bovine epitheliochorial placenta for maternal antibodies. Passive immunity is achieved through colostrum uptake and the absorption of the maternal immunoglobulins (**Ig**), mainly IgG, across the enterocytes of the small intestine via pinocytosis (Weaver et al., 2000; McGuirk and Collins, 2004). This process is time limited. A cessation of the absorption of Ig occurs approximately at 24 hours postnatum. An extension up to 36 hours postnatum is possible if feeding is delayed (Stott et al., 1979a; Weaver et al., 2000).

Despite maternal Ig, colostrum contains more nutrients and nonnutrient factors that are essential for the calves, such as protein, fat, lactose, vitamins, minerals, biologically active

peptides, growth factors, hormones, cytokines, and maternal leukocytes (McGuirk and Collins, 2004; Faber et al., 2005; Godden, 2008; Soberon et al., 2012; McGrath et al., 2016). The lactocrine hypothesis describes the effect of maternal milk-born factors transmitted over colostrum into the neonate through nursing (Bagnell et al., 2005; Bartol et al., 2008; Soberon et al., 2012). These milk-born factors including protein hormones, i.e. insulin, insulin-like growth factor I, epidermal growth factor, growth hormone, prolactin, leptin, and relaxin, influence the epigenetic development and growth of specific tissues, such as the gastrointestinal tract, liver, kidneys, spleen, and muscle. These hormones are available in higher concentrations in colostrum compared to the maternal blood levels. The milk-borne factors are absorbed in a similar fashion as the maternal Ig, through the neonatal enterocytes of the small intestine (Bagnell et al., 2005; Bartol et al., 2008).

Inadequate passive immunity or failure of passive transfer (**FPT**) is one of the major contributing factors to high morbidity and mortality rates of neonatal calves (Trotz-Williams et al., 2008; Beam et al., 2009; Vasseur et al., 2010; Vogels et al., 2013; Boccardo et al., 2016). Currently, the prevalence of FPT in neonatal calves is estimated to reach 35%. A nationwide survey in Germany conducted by McMorran (2006) found a prevalence of FPT of 38.8%, 403 out of 1,037 neonatal calves from 154

commercial farms had FPT. The NAHMS report from 1993 and 2007 recorded a prevalence of FPT from 20 to 40%, respectively, in neonatal calves in the US. Failure of passive transfer is defined as serum IgG concentrations below 10 mg/mL in 24 to 48 h old calves, whereas successful passive immunity is reached at serum IgG concentrations above 10 mg/mL (Weaver et al., 2000; McGuirk and Collins, 2004; Godden, 2008). It has been shown that calves suffering from FPT have a higher risk for infections and are more likely to develop illnesses, especially bovine respiratory diseases and diarrhea (Weaver et al., 2000; Raboisson et al., 2016). Furthermore, the perinatal mortality linked to FPT ranged from 8 to 25% (Raboisson et al., 2016). Thus, the impact of FPT is not restricted to the perinatal period. According to Robison et al. (1988) dairy heifers with adequate passive transfer had a significant increase in average daily gain in comparison to those with FPT ($P < 0.01$). Furthermore, a higher mortality rate in heifers with FPT was recorded, especially during the postweaning period (Robison et al, 1988).

The newborn calf care after parturition is crucial for a successful dairy calf management. A proper neonatal adaptation to extrauterine life is essential for the health and survival of the neonatal calf. Many factors influence the acquisition of passive immunity of neonatal calves. A large part is controllable with a good colostrum management. A quick harvesting of colostrum

under hygienic conditions and a timely feeding of the first meal postnatum, as well as an appropriate volume and quality of colostrum are essential to guarantee a successful passive transfer (Pritchett et al., 1991; Weaver et al., 2000; Godden, 2008). Furthermore, the abomasal emptying rate and a poor vigour of the neonate affect the apparent efficiency of absorption (**AEA**) of ingested IgG and therefore influence the passive immunity without giving momentarily the possibility of intervention, apart from a sound calving management to prevent dystocia (Barrier et al., 2011; Mokhber-Dezfooli et al., 2012).

Preventive strategies have been developed that are primarily focused on good colostrum management, which includes the nutrition of the dam during the transition period, a good calving management, the timely and hygienic harvesting of the first colostrum, quality control of colostrum, adequate feeding and drinking regimes of the neonatal calves, as well as the hygienic storage of the colostrum (Weaver et al., 2000; McGuirk and Collins, 2004; Godden, 2008). To ensure a good colostrum management, different control tools have to be established into the daily work routine of a dairy farm to evaluate the performance of colostrum management.

Failure of passive transfer and its consequences represent a major problem in the dairy industry. In order to optimize colostrum management and the rate of successful

passive transfer in neonatal calves, two recent studies have focused on benchmarking among dairy farms (Atkinson et al., 2017; Sumner et al. 2018). Benchmarking is used in other areas, such as in health care, farming and commercial industry, to compare one's own performance with another peer (Jarrar and Zairi, 2001; Bogetoft, 2012). This offers the possibility to reflect on current practices and identify sources of error (Meade, 1994; Anand and Kodali, 2008). Atkinson et al. (2017) evaluated the effects of benchmarking on the prevalence of FPT and rates of average daily gain in preweaned calves on dairy farms in Canada. Both studies concluded that benchmarking motivated farmers for management changes with the intention to improve performance.

On this account, the regular monitoring of FPT via blood samples of the calves should be implemented in order to evaluate the quality of colostrum management. The NAHMS report of 2014 recorded that only 6.2% of US dairy farms monitor FPT routinely. The laboratory methods, radial immunodiffusion (**RID**), the gold standard for FPT assessment, and enzyme-linked immunosorbent assay (**ELISA**), directly measure the serum IgG concentrations of neonatal calves. However, these tests are expensive, technically demanding, and time-consuming (Davis and Giguère, 2005; Hogan et al., 2015). On-farm devices measure indirectly the IgG concentration by assessment of the

total protein (**TP**) or total solids (**TS**) in serum or plasma using refractometry (Deelen et al., 2014; Elsohaby et al., 2019). For refractometry, a handheld optical refractometer can be used measuring the TP concentration (≥ 5.5 g/dL in serum), or a digital Brix refractometer to assess the TS percentage in % Brix (≥ 8.4% Brix). All methods, however, require centrifugation and are thus more difficult for farmers to implement as a calf-side monitoring tool. Therefore, we conducted two studies regarding the monitoring of FPT. The first study evaluated a disposable plasma filter system as a point-of-care device for FPT assessment. The second study focused on four different direct and indirect analytical methods for FPT assessment in comparison to the gold standard RID.

Regarding the monitoring of colostrum quality, there are different laboratory methods and on-farm devices available. Every colostrum should be examined for its quality to verify that it is suitable for the calf's first meal. The on-farm devices allow the rapid assessment of colostrum quality, such as the determination of TS by refractometry (≥ 22% Brix indicate good quality for Holstein Friesian cattle) or the assessment of the specific gravity with a hydrometer (> 1.050 marks good quality colostrum). Laboratory methods such as RID and ELISA assess directly the IgG concentration of colostrum. An IgG concentration above 50 g/L identifies good quality colostrum, and further on,

total bacterial and fecal coliform counts should not exceed 100,000 and 10,000 cfu/mL (Weaver et al., 2000; McGuirk and Collins, 2004; Godden, 2008). In contrast to these laboratory methods, the on-farm devices allow a cost-effective and rapid measurement of colostrum quality (Weaver et al., 2000; Godden, 2008).

A nationwide study in the US for evaluation of colostrum composition and quality was conducted by Morrill et al. (2012). Approximately 70.6% of the colostrum produced on dairy farms had IgG concentrations greater than the recommendation of 50 mg/mL, whereas only 54.8% had total bacterial counts less than 100,000 cfu/mL. Furthermore, only 39.4% of the produced colostrum met both criteria (Morrill et al., 2012).

It is well known that colostrum quality has an important impact on calf health, and yet it is challenging for dairy farms to collect sufficient amounts. Therefore, we wanted to conduct a study evaluating two different treatment procedures at the first milking after calving to increase colostrum quantity and to improve colostrum quality in dairy cows. We expected to develop a reliable strategy for farmers to produce good colostrum.

The overall objectives of this thesis were (1) to evaluate a filter system to harvest plasma for identification of failure of passive transfer in newborn calves, (2) to evaluate two different treatment procedures after calving to improve harvesting of high-

quantity and high-quality colostrum, and (3) to evaluate different analytical methods to assess failure of passive transfer in neonatal calves.

Publication I and II were published in the Journal of Dairy Science (Impact Factor: 3.082). The additional unpublished work has been formatted according to the Journal of Dairy Science guidelines and has recently been submitted.

2 PUBLICATION I

Evaluation of a filter system to harvest plasma for identification of failure of passive transfer in newborn calves.

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2.1 Abstract

The objective of this study was to evaluate a filter system to harvest plasma to assess failure of passive transfer (FPT) in newborn calves. Blood samples (n = 227) for serum and plasma harvesting were collected via jugular vein puncture from Holstein calves aged 1 to 7 d from 4 commercial dairy herds in Northeast Germany. Serum IgG concentrations were determined using a sandwich ELISA. Failure of passive transfer was defined as IgG concentrations < 10 mg/mL and used as a gold standard. One handheld optical refractometer (Euromex Holland, Arnhem, the Netherlands) and 2 digital Brix refractometers (device 1: HI 96801 digital refractometer, Hanna Instruments, Woonsocket, RI; device 2: Misco PA201, Misco, Solon, OH) were used to analyze total proteins in serum or plasma. The colostrum uptake of the calf can thus be monitored and calves with FPT can be identified. Serum was obtained through centrifugation. Plasma was obtained through either a filter system or centrifugation. For plasma filtration, approximately 2 mL of lithium heparin blood was injected into the inlet reservoir of a plasma filter (2-Drop-Filter, Pharmadoc, Lübeck, Germany) using a disposable syringe. Receiver operating characteristic curve analyses were used to determine optimum thresholds for each of the 3 devices using different media. Sixty-seven (30%) calves had FPT. For the

handheld optical refractometer, the optimum threshold was 5.6 g/dL [sensitivity 70.1%; specificity 80.0%; positive predictive value (PPV) 60.1%; negative predictive value (NPV) 86.2%; area under the curve (AUC) 0.85] using serum. For centrifuged plasma, the optimum threshold was 6.3 g/dL (sensitivity 82.1%; specificity 68.1%; PPV 52.5%; NPV 89.9%; AUC 0.84), and for filtered plasma, the threshold was 6.0 g/dL (sensitivity 56.7%; specificity 90.0%; PPV 70.9%; NPV 82.9%; AUC 0.80). For device 1, the optimum threshold was 8.9% Brix (sensitivity 82.1%; specificity 63.8%; PPV 48.7%; NPV 89.5%; AUC 0.81), 9.4% Brix (sensitivity 76.1%; specificity 73.7%; PPV 55.4%; NPV 87.8%; AUC 0.80), using serum and centrifuged plasma, respectively. For device 2, the optimum threshold was 8.7% Brix (sensitivity 74.6%; specificity 76.2%; PPV 57.4%; NPV 87.5%; AUC 0.83), 9.5% Brix (sensitivity 80.6%; specificity 70.6%; PPV 54.0%; NPV 89.5%; AUC 0.83), and 9.2% Brix (sensitivity 58.2%; specificity 87.5%; PPV 66.6%; NPV 83.0%; AUC 0.80) using serum, centrifuged plasma, and filtered plasma, respectively. Based on the AUC, the 3 devices yielded comparable test characteristics to identify calves with FPT. In conclusion, a filter system can be used to facilitate the evaluation of FPT as a point-of-care technique in calves without the need for serum centrifugation.

2.2 Key words

plasma filter, failure of passive transfer, colostrum calves

2.3 Introduction

Management and nutrition of the newborn calf on the first day of life are critically important in optimizing its immunological status and therefore its further health and performance. The quick and adequate intake of high-quality colostrum is essential for delivering sufficient maternal IgG, nutrients, growth factors, and other nonnutrient factors. Insufficient absorption of IgG by the neonatal calf have many negative consequences, such as increased risk of disease and death, slower growth rates, and a reduction in long-term productivity (Robison et al., 1988; DeNise et al., 1989). Furthermore, a quick and adequate colostrum supply has been shown to affect the metabolism and the future performance of the neonatal calf (Faber et al., 2005; Soberon et al., 2012). Inadequate supply of IgG may occur for many reasons, such as timing of the first feeding (Besser et al., 1985), volume of the feeding (Stott et al., 1979), and concentration of IgG in colostrum that was fed (Pritchett et al., 1991).

The paramount role of colostrum for supplying IgG to the neonatal calf has been thoroughly investigated and is well documented (Weaver et al., 2000; Godden, 2008). In utero the

bovine epitheliochorial placenta prevents the transfer of IgG from the dam to the calf. To acquire immunity, the newborn calf must absorb a critical mass of IgG from colostrum before cessation of intestinal transport occurs at 24 to 36 h of age (Weaver et al., 2000; Barrington and Parish, 2001). Therefore, it is essential to provide an adequate amount of high-quality colostrum during the first 2 h after birth (Godden, 2008).

Failure of passive transfer (**FPT**) in calves is defined as serum IgG concentration below 10 mg/mL (NAHMS, 1993; Weaver et al., 2000; Godden, 2008). The reported prevalence of FPT in other studies ranged from 4.75 to 37.1% (Wallace et al., 2006; Trotz-Williams et al., 2008; Beam et al., 2009; Morrill et al., 2013; Deelen et al., 2014).

Continuous monitoring of successful passive transfer in newborn calves should be a crucial component of colostrum management (Godden, 2017). Only 6.2% of US dairy farms routinely monitor FPT on a regular basis (NAHMS, 2014). There are tests available that evaluate serum IgG concentrations, such as radial immunodiffusion (**RID**) and ELISA, but these tests cannot be considered on farm and are technically demanding and costly (Davis and Giguère, 2005; Hogan et al., 2015). An alternative way to monitor FPT is via an indirect analysis method, the measurement of the total protein (**TP**) concentration in serum using refractometry (Deelen et al., 2014). Immunoglobulins

account for a large part of TP in neonatal calf serum; therefore, refractometry provides an approximation of the serum Ig concentration (Calloway et al., 2002). A handheld optical refractometer can be used for measuring the TP concentration (g/dL) in serum, or a digital Brix refractometer can be used to assess the total solids (**TS**) percentage in percentage Brix (% Brix). Both methods are highly correlated with the blood IgG concentration of calves determined by RID. Therefore, these simplified analysis methods allow a good estimation of IgG concentration (Deelen et al., 2014; Buczinski et al., 2018). Calves aged at least 24 h to 7 d can be tested (McGuirk and Collins, 2004). Thresholds to define successful passive transfer ranged from 4.6 to 5.8 g/dL of TP in serum (Lee et al., 2008; McCracken et al., 2017) and from 7.1 to 8.8% Brix measured with a digital Brix refractometer, respectively (Cuttance et al., 2017; McCracken et al., 2017).

For monitoring IgG or TP, it is necessary to separate the serum from the blood cells using a centrifuge. Therefore, its application as an on-farm test is limited. In human medicine, a filter system was developed for point-of-care devices to separate plasma from blood cells. This system was shown to be efficient in separating plasma from cells and has several advantages, such as being portable and quick and requiring only small amounts of blood (Crowley and Pizziconi, 2005; Chen et al.,

2009). This method, however, has not been validated for subsequent measurement of TP via refractometry in veterinary medicine.

The objective of this study was to evaluate a filter system to harvest plasma to assess FPT in newborn calves. We hypothesized that TP measured in filtered plasma using refractometry is highly correlated with serum IgG concentration and serum TP obtained by centrifugation.

2.4 Materials and Methods

2.4.1 Animals and Sampling

The experimental procedures reported herein were conducted with the approval of the Institutional Animal Care and Use Committee of the Freie Universität Berlin. Holstein Friesian calves from 4 commercial dairy herds in Northeast Germany were sampled between August and December 2017. Whole blood was collected from calves (n = 227) 1 to 7 d old by jugular venipuncture using a 20-gauge, 1.5-inch hypodermic needle (Vacutainer, Greiner Bio-One GmbH, Kremesmünster, Austria). Blood was collected into 2 sterile plastic Vacutainer tubes. One tube did not contain any anticoagulant (8.5 mL, BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ), and the other contained

lithium heparin (9 mL, Greiner Bio-One GmbH). Samples were stored on ice for transportation to the Freie Universität Berlin.

2.4.2 Filtration and Centrifugation

Within 4 to 6 h of collection, serum and heparinized plasma were separated by either centrifugation at 1,500 × *g* for 6 min at approximately 20°C or filtration. For plasma filtration, approximately 2 mL of lithium heparin blood was injected into the inlet reservoir of a plasma filter (2-Drop-Filter, Pharmadoc, Lübeck, Germany) using a disposable syringe (12 mL, Henry Schein, Langen, Germany). One filter was used for each measurement, and 2 drops of plasma were obtained per filter. Afterward, the lithium heparin blood samples were centrifuged to obtain plasma. Aliquots of serum and plasma were collected for further analysis using an optical refractometer (handheld refractometer, Euromex Holland, Arnhem, the Netherlands). One milliliter of serum was pipetted into a sterile vial (Cryovial 2 mL, Simport, Bernard-Pilon, Germany) and sent frozen to the Department of Veterinary Science, Chair of Animal Welfare, Ethology, Animal Hygiene and Animal Husbandry (Faculty of Veterinary Medicine, LMU Munich, Germany) for IgG testing.

Total protein of serum samples and centrifuged plasma samples was measured by 2 digital Brix refractometers in % Brix

(device 1: HI 96801 digital refractometer, Hanna Instruments, Woonsocket, RI; device 2: Misco PA201, Misco, Solon, OH) and by 1 handheld optical refractometer (Euromex Holland) assessing TP in grams per deciliter to evaluate FPT. Filtered plasma was analyzed with device 2 and the handheld optical refractometer. The measurement with device 1 was not possible because 2 droplets of plasma was not sufficient for the analysis. All 3 refractometers are precision optical devices designed to analyze the concentration of water-soluble solids. They measure the refractive index. The digital Brix refractometers additionally assess the speed at which light passes through the liquid. The denser the liquid, the slower the light passes through it. All devices implement temperature compensation automatically so that an accurate measurement is possible between 0 and 40°C for device 1, 0 and 50°C for device 2, and 0 and 30°C for the handheld refractometer. Calibration was performed with distilled water before each batch of samples.

2.4.3 IgG Analysis

The IgG analysis in serum measured by sandwich ELISA was conducted at the Department of Veterinary Science, Faculty of Veterinary Medicine, LMU Munich. The sandwich ELISA method was performed according to Erhard et al. (1999) and was

based on coating and conjugating the serum IgG with anti-bovine IgG coupled to a peroxidase enzyme (A 5295, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). This peroxidase catalyzed a color change, which was proportional to the IgG concentration of the sample. The quantification was measured photometrically at 450 nm by an ELISA reader (GENios, Tecan Germany GmbH, Crailsheim, Germany).

Into each well of a 96-well polystyrene plate (F96 Cert. Maxisorp Nunc-Immuno Plate, Thermo Scientific GmbH, Darmstadt, Germany) rabbit serum with anti-bovine IgG (5 µg/mL; A 5645, Sigma-Aldrich Chemie GmbH) was transferred and blocked with a 0.5% gelatin PBS solution. At the uppermost well of each column, the serum samples were inoculated and diluted with PBS-Tween buffer to obtain 50 µL in each well. Furthermore, the peroxidase-linked rabbit anti-bovine IgG was pipetted into each well. The substrate solution [332 µL of stock solution, 10 mL of 3.3',5.5'-tetramethylbenzidine (**TMB**) buffer, 30% H_2O_2] was added, whereby the enzyme reaction took place. By applying 50 µL of 1 *M* sulfuric acid, the reaction process was stopped and the photometric intensity was measured. The mean value of each column and thus of each serum sample resulted in the final IgG concentration (mg of IgG/mL).

2.4.4 Statistical Analysis

The TP (in g/dL) and refractometry results (in % Brix) from centrifuged or filtered plasma were plotted against the IgG concentration (in mg/mL) in centrifuged serum. From these distribution plots, correlation coefficients (r-values) were determined. The comparison against centrifuged serum was performed only for the handheld optical refractometer and the second digital Brix device (Misco PA201), whereas the comparison against IgG concentration included all 3 devices. The average (means ± SD) for all the different media and for IgG concentration was calculated using MedCalc software (version 15.6.1, MedCalc, Mariakerke, Belgium). Test characteristics [sensitivity, specificity, positive predictive value (**PPV**), and negative predictive value (**NPV**)] were calculated using MedCalc software (version 15.6.1, MedCalc) for all 3 devices and the different media (i.e., centrifuged serum, centrifuged plasma, filtered plasma). Sensitivity was defined as the probability of a test result correctly indicating FPT for a sample with IgG < 10 mg/mL. Specificity was defined as the probability of a test result correctly indicating adequate passive transfer for a sample with IgG ≥ 10 mg/mL. The PPV was defined as a predictive probability of a test result correctly indicating FPT for a sample with IgG < 10 mg/mL. The NPV was defined as a predictive

probability of a test result correctly indicating adequate passive transfer for a sample with IgG ≥ 10 mg/mL.

A receiver operating characteristic (**ROC**) curve was created to plot the true-positive rate against the false-positive rate at 0.1 g/dL and 0.1% Brix increments, respectively. The critical threshold was assessed with an ROC curve. The point on the curve with the highest combined sensitivity and specificity was considered as the critical threshold. According to Swets (1988) the interpretation of this critical threshold is based on the area under the curve (**AUC**) as perfect (AUC = 1), highly accurate (0.9 < AUC < 1), very accurate (0.7 < AUC < 0.9), accurate (0.5 < AUC < 0.7), and non-informative (AUC = 0.5). Variables were declared as significant statistical difference between the levels of a classification when $P < 0.05$; a statistical tendency was defined as differences between $P \geq 0.05$ and $P \leq 0.10$.

2.5 Results

Out of 227 calves, 67 (30%) had serum IgG concentrations < 10 mg/mL and 160 (70%) had serum IgG concentrations ≥ 10 mg/mL. The mean IgG concentration was 18.4 ± 11.6 mg/mL, and the range was 1.1 to 55.5 mg of IgG/mL (Table 1).

The mean % Brix in serum in the present study for device 1 (HI 96801) was 9.0 ± 0.85% Brix, and the range was 6.9 to 11.6% Brix (Table 1). For centrifuged plasma, the mean was 9.6 ± 0.80% Brix and the range was 7.7 to 12.4% Brix. For device 2 (Misco PA201), the mean was 9.0 ± 0.87% Brix for serum, 9.6 ± 0.83% Brix for centrifuged plasma, and 9.8 ± 0.86% Brix for filtered plasma. The assessment via handheld optical refractometer resulted in a mean TP concentration of 5.9 ± 0.68 g/dL in serum, 6.4 ± 0.66 g/dL in centrifuged plasma, and 6.5 ± 0.68 g/dL in filtered plasma. Descriptive statistics for % Brix and TP in grams per deciliter are summarized in Table 1.

The correlation coefficients and the coefficients of determination are summarized in Figures 1, 2, 3, and 4. The readings from the handheld optical refractometer and the digital refractometer (device 2, Misco PA201) were highly correlated using centrifuged serum, centrifuged plasma, or filtered plasma (Figure 1). The range of correlation coefficients was r = 0.87 to 0.92 (Figure 1). The correlation between the IgG concentration in milligrams per milliliter and the handheld optical refractometer was r = 0.58 in serum (Figure 2), r = 0.51 in centrifuged plasma (Figure 3), and r = 0.47 in filtered plasma (Figure 4). The correlation of IgG concentration in milligrams per milliliter and device 1 (HI 96801) was r = 0.50 (Figure 2) and in centrifuged plasma was r = 0.43 (Figure 3). Furthermore, the correlation of

IgG concentration and device 2 (Misco PA201) was r = 0.55 in serum, r = 0.49 in centrifuged plasma, and r = 0.46 in filtered plasma (Figures 2, 3, and 4).

Test characteristics (optimum thresholds, sensitivity, specificity, PPV, NPV, and AUC) for the handheld optical and digital refractometers to identify calves with FPT using 3 different media were determined by ROC curve analyses (Table 2). For the handheld optical refractometer, the optimum threshold was 5.6 g/dL with a sensitivity of 70.1%, specificity of 80.0%, PPV of 60.1%, NPV of 86.2%, and AUC of 0.85. For device 1 (HI 96801), the optimum threshold using serum was 8.9% Brix (sensitivity 82.1%, specificity 63.8%, PPV 48.7%, NPV 89.5%, AUC 0.81; Table 2). For device 2 (Misco PA201), the optimum threshold measured in serum was 8.7% Brix (sensitivity 74.6%, specificity 76.2%, PPV 57.4%, NPV 87.5%, AUC 0.83). In general, optimal thresholds for identifying calves with FPT using plasma were greater compared with serum irrespective of the method of plasma harvesting. The 3 different devices had comparable AUC irrespective of the medium used.

Table 1. Descriptive statistics for total protein (g/dL), percentage points Brix (% Brix) and IgG concentration (mg IgG/mL) considering centrifuged serum, centrifuged plasma, and filtered plasma.

Test[1]	Medium	n	Mean	SD[2]	Minimum	Maximum
Misco Brix refractometer	Centrifuged serum	227	9.0% Brix	0.87	7.0	11.6
	Centrifuged plasma	227	9.6% Brix	0.83	7.7	12.5
	Filtered plasma	227	9.8% Brix	0.86	7.6	12.5
Hanna Brix refractometer	Centrifuged serum	227	9.0% Brix	0.85	6.9	11.6
	Centrifuged plasma	227	9.6% Brix	0.80	7.7	12.4
Handheld optical refractometer	Centrifuged serum	227	5.9 g/dL	0.68	4.4	8.0
	Centrifuged plasma	227	6.4 g/dL	0.66	4.8	8.6
	Filtered plasma	227	6.5 g/dL	0.68	4.9	8.8
Sandwich ELISA	Centrifuged serum	227	18.4 mg IgG/mL	11.6	1.1	55.5

[1]Misco PA201 Brix refractometer: Misco, Solon, Ohio; HI 96801 Brix refractometer: Hanna Instruments, Woonsocket, Rhode Island; handheld optical refractometer: Euromex Holland, Arnhem, the Netherlands.

[2]SD = Standard deviation of the estimate.

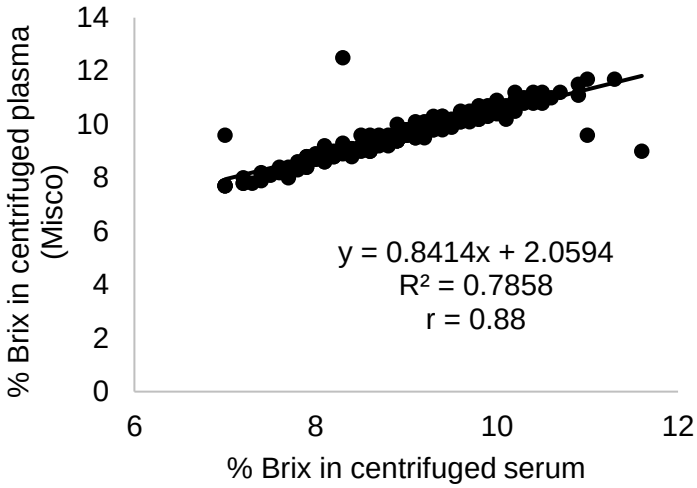
Table 2. Test characteristics for total protein (g/dL) and percentage points Brix (% Brix) for identification of calves with failure of passive transfer (FPT; IgG < 10.0 mg/mL) 1 to 7 days of age using 3 different media.

Device[2]	Medium	n	Threshold[3]	AUC[1] (95% CI)	*P*-value	Sensitivity	Specificity	PPV[1] (95% CI)	NPV[1] (95% CI)
Misco Brix refractometer	Centrifuged serum	227	8.7% Brix	0.83 (0.78 – 0.88)	0.001	74.6	76.2	57.4 (45.8 – 67.3)	87.5 (81.1 – 92.7)
	Centrifuged plasma	227	9.5% Brix	0.83 (0.77 – 0.88)	0.001	80.6	70.6	54.0 (43.3 – 63.5)	89.5 (83.0 – 94.4)
	Filtered plasma	227	9.2% Brix	0.80 (0.74 – 0.85)	0.001	58.2	87.5	66.6 (52.5 – 78.0)	83.0 (76.8 – 88.6)
Hanna Brix refractometer	Centrifuged serum	227	8.9% Brix	0.81 (0.75 – 0.86)	0.001	82.1	63.8	48.7 (39.2 – 58.3)	89.5 (82.3 – 94.5)
	Centrifuged plasma	227	9.4% Brix	0.80 (0.74 – 0.85)	0.001	76.1	73.7	55.4 (44.1 – 65.2)	87.8 (81.3 – 93.0)
Handheld optical refractometer	Centrifuged serum	227	5.6 g/dL	0.85 (0.80 – 0.89)	0.001	70.1	80.0	60.1 (47.8 – 70.5)	86.2 (79.9 – 91.6)
	Centrifuged plasma	227	6.3 g/dL	0.84 (0.79 – 0.89)	0.001	82.1	68.1	52.5 (42.0 – 61.7)	89.9 (83.3 – 94.8)
	Filtered plasma	227	6.0 g/dL	0.80 (0.74 – 0.85)	0.001	56.7	90.0	70.9 (56.2 – 82.1)	82.9 (76.8 – 88.5)

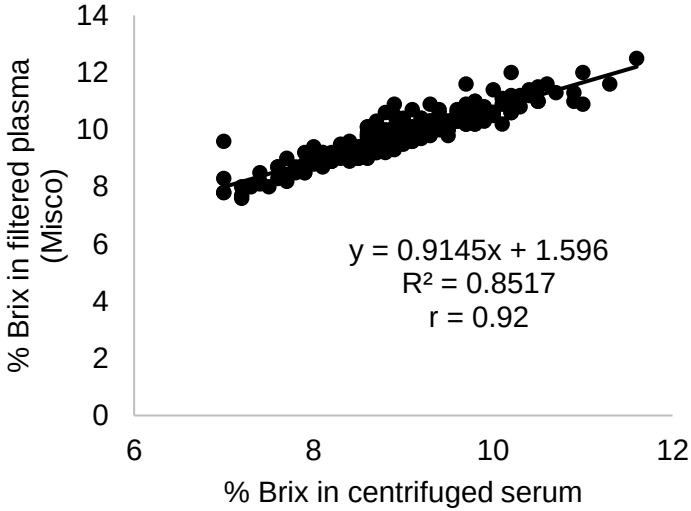
[1]AUC= area under the curve; PPV = positive predictive value; NPV = negative predictive value.

[2]Misco PA201 Brix refractometer: Misco, Solon, Ohio; HI 96801 Brix refractometer: Hanna Instruments, Woonsocket, Rhode Island; handheld optical refractometer: Euromex Holland, Arnhem, the Netherlands.

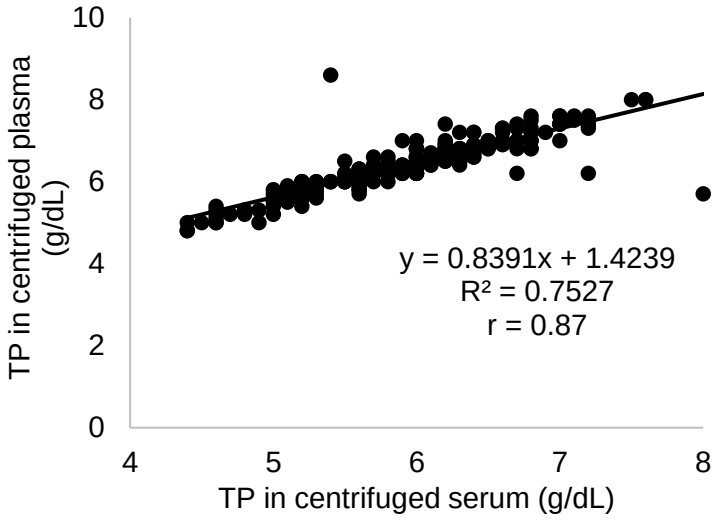
[3]Optimal threshold was determined by receiver operating characteristic curve analysis using the threshold with the highest sum of sensitivity and specificity to identify calves with failure of passive transfer.


% Brix in centrifuged plasma (Misco)
14
12
10
8
6
4
2
0
6
8
10
12
% Brix in centrifuged serum
y = 0.8414x + 2.0594
R² = 0.7858
r = 0.88

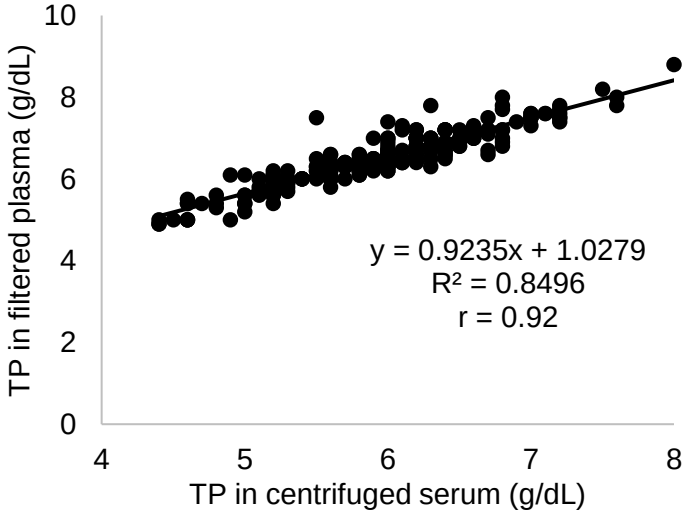
a)


% Brix in filtered plasma (Misco)
14
12
10
8
6
4
2
0
6
8
10
12
% Brix in centrifuged serum
y = 0.9145x + 1.596
R² = 0.8517
r = 0.92

b)

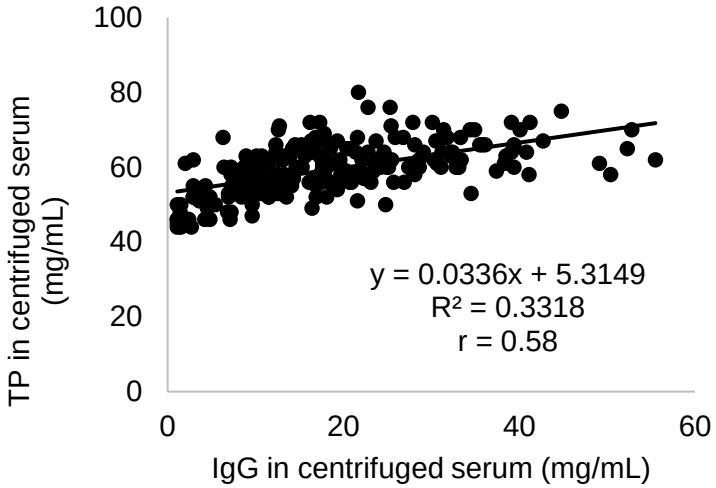

10
8
6
4
2
0
4
5
6
7
8
TP in centrifuged plasma (g/dL)
TP in centrifuged serum (g/dL)
y = 0.8391x + 1.4239
R² = 0.7527
r = 0.87

c)

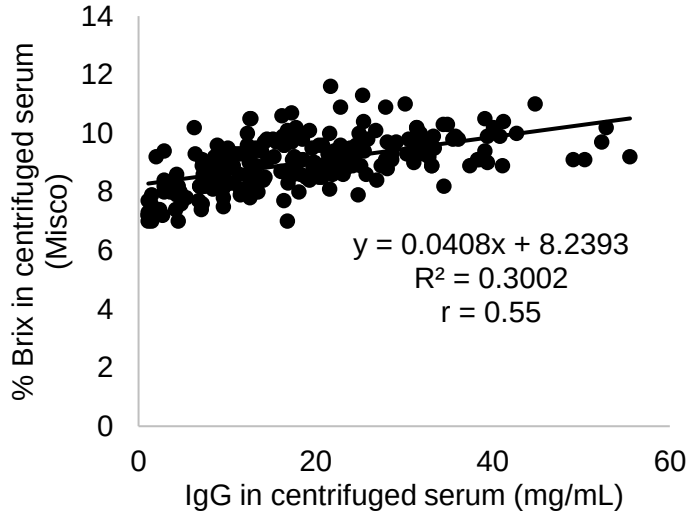

10
8
6
4
2
0
4
5
6
7
8
TP in filtered plasma (g/dL)
TP in centrifuged serum (g/dL)
y = 0.9235x + 1.0279
R² = 0.8496
r = 0.92

d)

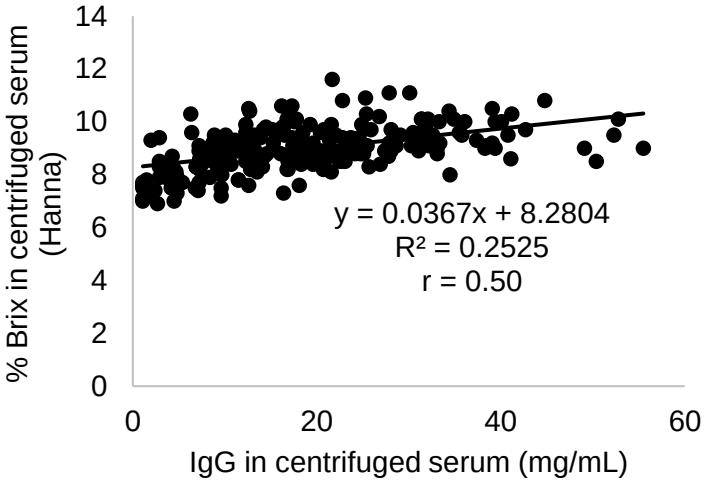
Figure 1. a) Total solids (TS) concentration in centrifuged serum compared with TS in centrifuged plasma of calves using the Misco PA201 Brix refractometer (Misco, Solon, OH; n = 227; r = 0.88); **b)** TS concentration in centrifuged serum compared with TS in filtered plasma of calves using the Misco PA201 Brix refractometer (n = 227; r = 0.92); **c)** Total protein (TP) concentration in centrifuged serum compared with TP in centrifuged plasma of calves using the handheld optical refractometer (n = 227; r = 0.87); **d)** TP concentration in centrifuged serum compared with TP in filtered plasma of calves using the handheld optical refractometer (n = 227; r = 0.92). % Brix = percentage points Brix.


100
80
60
40
20
0
TP in centrifuged serum (mg/mL)
y = 0.0336x + 5.3149
R² = 0.3318
r = 0.58
0
20
40
60
IgG in centrifuged serum (mg/mL)

a)

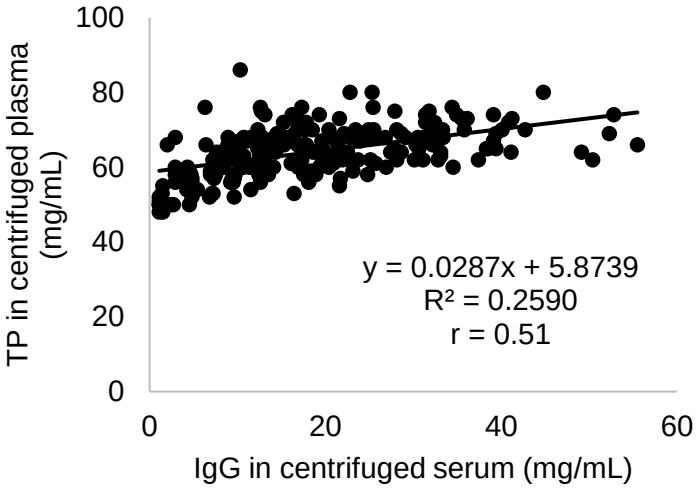

14
12
10
8
6
4
2
0
% Brix in centrifuged serum (Misco)
y = 0.0408x + 8.2393
R² = 0.3002
r = 0.55
0
20
40
60
IgG in centrifuged serum (mg/mL)

b)

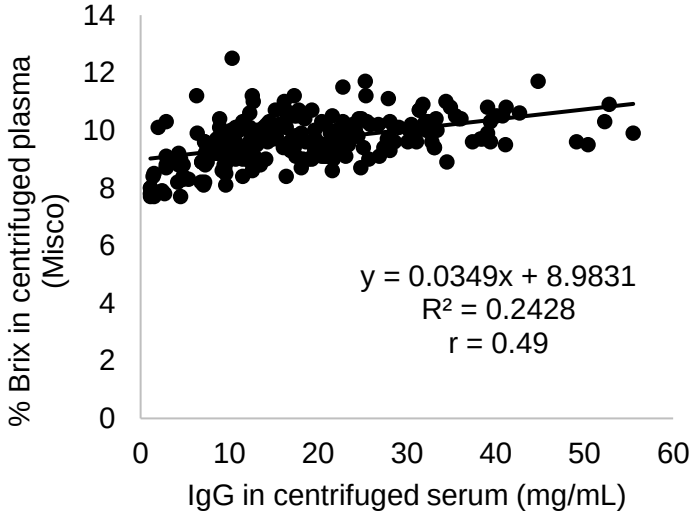


c)

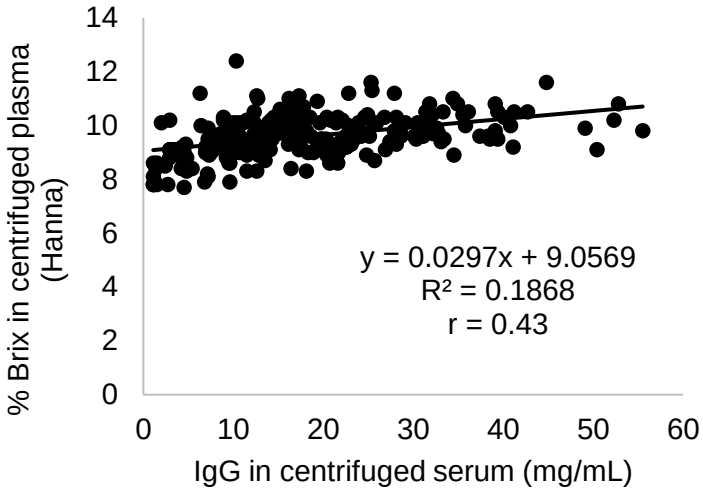
Figure 2. a) Total protein (TP) concentration in centrifuged serum using the handheld optical refractometer compared with IgG concentration measured by ELISA in centrifuged serum of calves (n = 227; r = 0.58); **b)** percentage points Brix (% Brix) using the Misco PA201 Brix refractometer (Misco, Solon, OH) in centrifuged serum compared with IgG concentration measured by ELISA in centrifuged serum of calves (n = 227; r = 0.55); **c)** % Brix using the HI 96801 Brix refractometer (Hanna Instruments, Woonsocket, OH) in centrifuged serum compared with IgG concentration measured by ELISA in centrifuged serum of calves (n = 227; r = 0.50). % Brix = percentage points Brix.


100
80
60
40
20
0
TP in centrifuged plasma (mg/mL)
y = 0.0287x + 5.8739
R² = 0.2590
r = 0.51
0
20
40
60
IgG in centrifuged serum (mg/mL)

a)

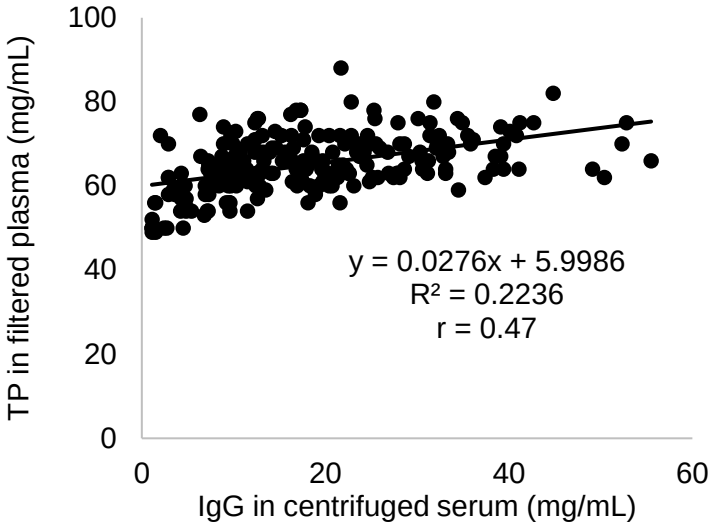

14
12
10
8
6
4
2
0
% Brix in centrifuged plasma (Misco)
y = 0.0349x + 8.9831
R² = 0.2428
r = 0.49
0
10
20
30
40
50
60
IgG in centrifuged serum (mg/mL)

b)

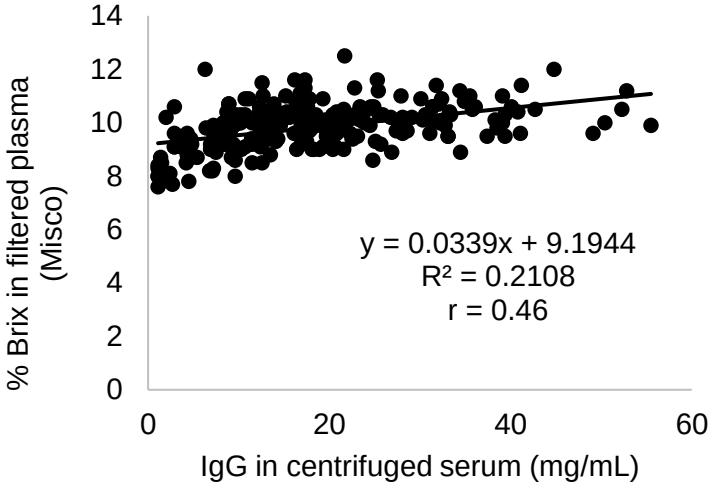


c)

Figure 3. a) Total protein (TP) concentration in centrifuged plasma using the handheld optical refractometer (Euromex Holland, Arnhem, the Netherlands) compared with IgG concentration measured by ELISA in centrifuged serum of calves (n = 227; r = 0.51); **b)** percentage points Brix (% Brix) using the Misco PA201 Brix refractometer (Misco, Solon, OH) in centrifuged plasma compared with IgG concentration measured by ELISA in centrifuged serum of calves (n = 227; r = 0.49); **c)** % Brix using the HI 96801 Brix refractometer (Hanna Instruments, Woonsocket, OH) in centrifuged plasma compared with IgG concentration measured by ELISA in centrifuged serum of calves (n = 227; r = 0.43). % Brix = percentage points Brix.


TP in filtered plasma (mg/mL)
100
80
60
40
20
0
0
20
40
60
IgG in centrifuged serum (mg/mL)
y = 0.0276x + 5.9986
R² = 0.2236
r = 0.47

a)


% Brix in filtered plasma (Misco)
14
12
10
8
6
4
2
0
0
20
40
60
IgG in centrifuged serum (mg/mL)
y = 0.0339x + 9.1944
R² = 0.2108
r = 0.46

b)

Figure 4. a) Total protein (TP) concentration in filtered plasma using the handheld optical refractometer (Euromex Holland, Arnhem, the Netherlands) compared with IgG concentration measured by ELISA in centrifuged serum of calves (n = 227; r = 0.47); **b)** percentage points Brix (% Brix) using the Misco PA201 Brix refractometer (Misco, Solon, OH) in filtered plasma compared with IgG concentration measured by ELISA in centrifuged serum of calves (n = 227; r = 0.46). % Brix = percentage points Brix.

2.6 Discussion

The objective of this study was to evaluate a filter system to harvest plasma to assess FPT in newborn calves. We hypothesized that TP and % Brix measured in filtered plasma using refractometry are highly correlated with serum IgG concentration and therefore suitable for identifying calves with FPT. Thirty percent (67/227) of the animals had serum IgG < 10 mg/mL, representing FPT. The prevalence of FPT in our study was comparable with other studies using the same definition of FPT and similar age of calves (Lee at al., 2008; Morrill et al., 2013; Elsohaby et al., 2015). This study is, however, not representative; hence, the participating farms represent a convenience sample because they were not randomly selected.

The correlation between serum TP and IgG determined by ELISA in our study was lower (r = 0.58) than previously reported (Deelen et al., 2014; Elsohaby et al., 2015; Hernandez et al., 2016; Cuttance et al., 2017). Correlation analysis revealed similar results for TP in centrifuged (r = 0.51) and filtered (r = 0.47) plasma compared with IgG concentration assessed by sandwich ELISA. Those studies, however, used RID or turbidimetric immunoassay for IgG analysis instead of sandwich ELISA. The correlation between % Brix and IgG determined by sandwich ELISA in our study was also lower (device 1: r = 0.50;

device 2: r = 0.55) than in other studies (Morrill et al., 2013; Deelen et al., 2014; Elsohaby et al., 2015; Hernandez et al., 2016; Cuttance et al., 2017). Two recent studies evaluated the agreement between an ELISA and RID to analyze IgG in serum and plasma of calves and found only weak to moderate correlations (Gelsinger et al., 2015; Dunn et al., 2018). According to Gelsinger et al. (2015), the concentration of IgG was significantly lower in plasma and colostrum samples when measured by ELISA compared with RID, and their study results indicated that the RID is a more consistent method. The cutoff value from RID measurements for successful passive transfer (10 mg of IgG/mL) was established for serum and plasma samples (Tyler et al., 1996). Gelsinger et al. (2015) referenced that results from ELISA were significantly correlated with RID values, but a direct comparison of ELISA and RID values is not recommended due to a consistent underestimation of IgG concentration by ELISA compared with RID. The authors emphasized that a new threshold should be attributed for ELISA testing to determine FPT in newborn calves.

This might be a reason for the weak association of TP and % Brix measured in serum and in centrifuged and filtered plasma compared with serum IgG concentration measured with sandwich ELISA. The results generated in our study by ELISA limit comparability with other studies using RID. The single RID

measurement includes an agarose gel plate containing antiserum against bovine IgG. The sample spreads from the wells into the agarose gel by diffusion. When antigen and matching antibodies meet, they react with each other and form a precipitate ring. The size of the ring depends on the amount of antigen contained in the solution applied. The diameters of the precipitate rings were measured using a magnifying loupe and were plotted against the IgG concentrations of the standards using linear regression (Dunn et al., 2018). Disadvantages are a low antigen concentration that reduces accuracy and an uneven spread that can confound results. For the comparability and reproducibility of the results, it is crucial to measure the precipitate rings exactly at the same time (Varley et al., 1985). Because the ELISA is similar to RID in accuracy (Weaver et al., 2000), we chose ELISA as the gold standard.

Our methodology, however, does not limit our ability to compare different devices and media using the same gold standard to assess FPT. Moreover, the correlation of the serum IgG concentration using RID with the serum TP has already been sufficiently investigated and confirmed (Calloway et al., 2002; Moore et al., 2009; Deelen et al., 2014). Using the AUC as an overall indicator of test quality, the results of the 3 different devices and media are comparable because the 95% confidence interval for the AUC overlap. In addition, there was a high

correlation between centrifuged serum and filtered plasma using either the handheld optical refractometer or the digital Brix refractometer. These high correlations indicate that filtered and centrifuged plasma are well suited for determining FPT in calves.

The AUC was used as an overall indicator of test characteristics irrespective of the device or the medium and ranged from 0.8 to 0.85 in our study. The results, independent of the device or the medium, proved to be very accurate ($0.7 < AUC < 0.9$; Swets, 1988). This is comparable with other studies using a handheld optical refractometer (Lee et al., 2008; Elsohaby et al., 2015; Hernandez et al., 2016; Cuttance et al., 2017) or a digital Brix refractometer (Morrill et al., 2013; Elsohaby et al., 2015; Hernandez et al., 2016; Cuttance et al., 2017) to assess FPT in calves.

The optimum threshold in our study for identifying calves with FPT using serum TP was 5.6 g/dL and 8.7% Brix, and 8.9% Brix for devices 1 and 2, respectively. This is comparable with other studies (Lee et al., 2008; Elsohaby et al., 2015; Hernandez et al., 2016; Cuttance et al., 2017) using serum IgG concentration as a gold standard to define FPT and an ROC curve analysis to determine the best threshold. Using 5.6 g/dL as the threshold, sensitivity was 70.1%, specificity was 80.0%, PPV was 60.1%, and NPV was 86.2%. This is in line with a recent meta-analysis (Buczinski et al., 2018) in which sensitivity and

specificity were 88.2 and 77.9%, respectively, using 5.5 g/dL as a threshold in a population of calves under 14 d of age. However, Elsohaby et al. (2015) and Cuttance et al. (2017) reported greater overall test characteristics (AUC = 0.90, Elsohaby et al., 2015; AUC = 0.95, Hernandez et al., 2016; AUC = 0.94, Cuttance et al., 2017) compared with our study (AUC = 0.85 for handheld optical refractometer, 0.81 for device 1, and 0.83 for device 2). The reason for this difference remains speculative.

The PPV ranged from 48.7 to 70.9% considering different media and devices and a prevalence of 30% FPT. This might lead to overdiagnosing FPT. In filtered plasma the sensitivity was lower (56.7% for handheld optical refractometer; 58.2% for device 2) than in centrifuged plasma or serum. The reason for this difference remains again speculative and requires further research. Specific thresholds, however, for serum and plasma have to be used. In general, thresholds for plasma were higher compared with serum, most likely because of the presence of coagulation proteins in plasma, which are missing in serum. Fibrinogen represents a part of the protein fraction, which is captured during clotting (Lumeij and Maclean, 1996).

2.7 Conclusion

In conclusion, a filter system to facilitate FPT assessment as a point-of-care analysis in calves without the need to centrifuge serum would be beneficial for dairy farms because of the easy handling and cost reduction. This study is the first to report an approach that allows omitting centrifugation, a costly and impractical method for dairy farms limiting the ability to use TP or % Brix as a calf-side test. To recommend this filter system as a point-of-care system, further research is warranted.

2.8 Acknowledgements

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3 PUBLICATION II

Evaluation of 2 different treatment procedures after calving to improve harvesting of high-quantity and high-quality colostrum

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3.1 Abstract

The objective of this study was to evaluate 2 different treatment procedures at the first milking after calving to increase colostrum quantity and to improve colostrum quality in dairy cows. We hypothesized that either exogenous treatment with oxytocin or the presence of the calf at first milking would lead to higher colostrum quantity and higher IgG concentration. The study was conducted from October to December 2017 on a commercial dairy farm in Germany. A total of 567 cows at the time of calving were enrolled, but for the final analyses only 521 animals were considered. The cows were randomly assigned on a daily basis into 1 of 3 groups: (1) control group (n = 177), (2) application of 20 IU of oxytocin i.m. (OXY; n = 163), and (3) presence of the calf (CA; n = 181) before and during milking. Cows in the control and oxytocin group had no contact with their calves after calving and were milked in a separate milking parlor. Cows in the oxytocin group were injected with 20 IU of oxytocin i.m. 3 min before manual stimulation. For cows in the third group, the calf was placed into a calf cart and located in front of the cow 3 min before manipulation of the cow. Colostrum quantity was determined by a digital hanging scale. The colostrum quality was assessed with digital Brix refractometry and ELISA. To evaluate the effect of 2 different treatment procedures, a generalized

linear mixed model was constructed using SPSS (SPSS Inc., IBM, Ehningen, Germany). The mean colostrum quantity was 4.17 ± 0.30 kg (mean ± SE). The treatment procedures and the harvesting time after calving had no effect on colostrum quantity. Parity, calf birth weight, and calving time affected colostrum quantity. Cows in second parity had the lowest quantity of colostrum (3.74 ± 0.37 kg) compared with cows in parity 1 (4.75 ± 0.34 kg) and cows in parity 3 or greater (4.75 ± 0.38 kg). Cows calving during the night (22:00 until 06:00 h; 4.93 ± 0.37 kg) had the highest quantity of colostrum compared with cows calving in the morning (06:00 until 14:00 h; 4.17 ± 0.38 kg) or afternoon (14:00 until 22:00 h; 4.14 ± 0.34 kg). Regarding colostrum quality, 48% of the colostrum samples contained ≥ 50 mg of IgG/mL. The mean IgG concentration was 54.6 ± 2.80 mg of IgG/mL. Colostrum quality was affected by the treatment procedures, colostrum quantity, parity, calving time, harvesting time after calving, and the calving day during the week. Both treatment procedures (i.e., OXY with mean IgG concentration results of 57.0 mg of IgG/mL and CA with 56.0 mg of IgG/mL) resulted in higher IgG concentrations in colostrum compared with the control group (50.7 mg of IgG/mL). With increasing colostrum quantity, the colostrum quality decreased in primiparous and multiparous cows. A longer time lag between calving and milking negatively affected the colostrum quality.

Concentration of IgG was higher for cows in parity 3 or greater (64.6 ± 2.59 mg of IgG/mL) compared with cows in parity 1 (48.5 ± 2.86 mg of IgG/mL) and cows in parity 2 (50.7 ± 2.89 mg of IgG/mL). Cows calving during the night had greater IgG concentrations (60.4 ± 2.92 mg of IgG/mL) compared with cows calving in the morning (51.9 ± 2.98 mg of IgG/mL) or afternoon (51.3 ± 2.71 mg of IgG/mL). Harvesting colostrum on quieter days, such as Sundays, resulted in higher IgG concentrations (61.4 ± 3.70 mg of IgG/mL). The assessment by Brix refractometry resulted in a mean result of 26.0 ± 0.20% Brix. Treatment procedures and the harvesting time after calving had no effect on colostrum quality. A negative association was observed between colostrum quantity and quality in primiparous and multiparous cows determined by Brix refractometry. Brix readings were greater for cows in parity 3 or higher (27.7 ± 0.26% Brix) compared with cows in parity 1 (25.3 ± 0.30% Brix) and cows in parity 2 (25.0 ± 0.32% Brix). In conclusion, the treatment procedure for the first milking is irrelevant to improve the quantity of colostrum. Both treatment procedures, however, increased IgG concentrations as determined by ELISA.

3.2 Key words

dairy cow, colostrum management, colostrum quality, colostrum quantity, oxytocin, calf

3.3 Introduction

Management and nutrition of the newborn calf during the first hours of life have the potential to permanently affect the lifetime performance of a dairy cow (Faber et al., 2005; Soberon et al., 2012). The role of colostrum in supplying IgG to the neonatal calf has been well described (Weaver et al., 2000; Godden, 2008). It is known that the timely delivery of colostrum, colostrum quality and quantity, and rate and amount of intestinal IgG absorption are essential components to guarantee a successful passive transfer in calves (Godden, 2017; Kertz et al., 2017). To achieve these goals, it is important to harvest a sufficient quantity of high quality colostrum. Release of oxytocin is the prerequisite for milk ejection and complete colostrum harvest. A continuous ejection of colostrum is dependent on the presence of adequate circulating oxytocin concentration (Wellnitz and Bruckmaier, 2001).

Milk ejection is an innate neuroendocrine reflex, which involves the hypothalamus, pituitary gland, and sensory neurons in the teat. In neurosecretory terminals of the pituitary gland,

oxytocin is stored and emptied into the bloodstream upon successful stimulation (Bruckmaier and Blum, 1996; Tančin et al., 2001; Mačuhová et al., 2004). Tactile stimulation of the teats results in the release of oxytocin and causes the contraction of myoepithelial cells around the mammary alveoli, whereby the alveolar milk fraction can be removed (Bruckmaier and Blum, 1998; Tančin et al., 2001). While tactile stimulation of the teats is the primary sensory impulse for milk ejection, genital stimulation and the presence of the newborn calf are also potent stimuli for oxytocin release (Bruckmaier and Blum, 1998; Tančin et al., 2001). Furthermore, it was shown that the presence of the calf during machine milking could influence the release of oxytocin and milk ejection (Akers and Lefcourt, 1982; de Passillé et al., 1997; Lupoli et al., 2001).

The objective of this study was to evaluate 2 different treatment procedures at the first milking after calving to increase colostrum quantity and improve colostrum quality by applying exogenous oxytocin or to stimulate endogenous oxytocin secretion. We hypothesized that either exogenous treatment with oxytocin or the presence of the calf at first milking leads to higher colostrum quantity and higher IgG concentration.

3.4 Materials and Methods

3.4.1 Dairy Farm, Animals, and Milking

This study was conducted from October to December 2017 on a commercial dairy farm in Mecklenburg-Vorpommern, Germany, milking approximately 2,500 Holstein cows. The average annual milk yield was 11,000 kg/cow. From drying off to the first 20 DIM, cows were housed in a naturally ventilated transition management facility providing freestall barns bedded with sand and ad libitum access to feed and water. Heifer and cow pens had 36 and 144 stalls, respectively. Cows were fed a TMR diet once daily consisting of corn silage and grass silage as forage with corn and canola-meal based concentrate formulated to meet or exceed the dietary requirements for dry and lactating Holstein cows (NRC, 2001). The approximate intake of MP was estimated at 1,189 g/d. All prepartum cows were vaccinated during the dry period and prepartum heifers before the first calving to improve colostrum quality. The vaccination against Escherichia coli, bovine rotavirus and coronavirus (Rotavec Corona, MSD Animal Health, Intervet Deutschland GmbH, Unterschleißheim, Germany) was carried out 3 to 12 wk before calving. Postpartum cows were milked 3 times daily (06:00, 14:00, and 22:00 h).

3.4.2 Calving Management

Pregnant heifers and cows were moved on a weekly basis to the prepartum pen 21 d (± 5) before expected parturition. Prepartum cows and heifers were monitored every 30 min to detect signs of imminent parturition (i.e., restlessness, vaginal discharge with bloody traces, lying lateral with abdominal contractions, a visible or broken amniotic sac, or feet of the emerging calf outside the vulva). Animals were moved into an individual maternity pen (3.5 × 3.5 m) bedded with fresh straw when the amniotic sac was visible or broken outside the vulva, or appearance of feet of the emerging calf were detected outside the vulva. A vaginal examination was conducted in every animal transferred to the maternity pen to assess dilatation of the vulva and cervix, as well as position, posture, presentation, and vitality of the calf. If the cow had not delivered the calf 1 h after the appearance of the amniotic sac or calf feet outside the vulva, calving assistance was provided to reduce calf losses (Schuenemann et al., 2011). Intensity of calving assistance was recorded using a 4-point scale (0 = calving in the prepartum pen; 1 = no assistance; 2 = assistance by 1 person; 3 = assistance by at least 2 persons). Twins, caesarean sections, and stillbirths were recorded separately.

For vaginal examination and calving assistance, cows were restrained in headlocks. The cow's perineum was

thoroughly cleaned using warm water and a 10% tincture of iodine solution (Braunol, B. Braun Melsungen AG, Melsungen, Germany). Lubricant (MS Lubricant, MS Schippers GmbH, Kerken, Germany) was applied generously to the obstetrical gloves and the cow's vagina before performing the exam or providing assistance. After calving, calves were separated from their dam before any suckling occurred. Calves were weighed with an electronic scale (WA200 mobile platform scale, Meier-Brakenberg GmbH & Co. KG, Extertal, Germany). All newborn calves had their navels dipped using a 10% tincture of iodine solution (Braunol, B. Braun Melsungen AG) and were placed into a hutch (1.5 × 1 m) bedded with fresh straw for the first 24 h following birth. Approximately 30 min after calving, 4 L of pasteurized (Perfect Udder, Dairy Tech Inc., Greeley, CO), pooled, high quality colostrum were fed using an esophageal tube feeder (Dairymac Drencher, Dairytop, Beilen, the Netherlands). Colostrum containing ≥ 22% Brix was regarded as high quality colostrum (Bielmann et al., 2010). Calves were identified using a 12-digit unique ear tag. Cows had no visual contact with their calves once they were removed from the maternity pen except for cows allocated to group 3 (presence of the calf).

3.4.3 Animal Enrollment and Harvest of Colostrum

Primiparous and multiparous cows (n = 567) at the time of calving had to meet the following specific inclusion criteria (i.e., clinically healthy, unchanged colostrum, correct assignment to 1 of 3 groups according to the randomization plan) and exclusion criteria (i.e., milk fever, lame cows, bloody or mastitic colostrum, gestation length < 265 d, cows with birth of twins or caesarean section or fetotomy) to be enrolled or withdrawn, respectively. Forty-six animals were excluded due to multiple reasons. For the final analyses, 521 animals (365 multiparous cows and 156 first-calf heifers) were considered.

Prepartum dams were randomly assigned on a daily basis into 1 of 3 treatment procedures to harvest colostrum: (1) control group (**CON**, n = 177), (2) oxytocin group (**OXY**, n = 163) with application of 20 IU of oxytocin, and (3) presence of the calf before and during milking (**CA**, n = 181). Before initiation of the study, a list of prepartum dams was created to assign 1 of the 3 treatment procedures to a specific calving date using a random function of Excel (Office 2010, Microsoft Deutschland Ltd., Munich, Germany). During 24 h each calving was subjected to the treatment procedure which was assigned to that specific day by the Excel list. Each treatment procedure (CON, OXY, or CA) was performed for a total of 28 nonconsecutive days.

Cows were placed in a self-locking chute immediately after parturition to harvest colostrum. Teats were predipped, forestripped, and dry wiped using a clean paper towel. Forestripping included the manual removal of 2 streams of colostrum from each teat. Manual stimulation lasted 30 s. The lag time between manual stimulation and attachment of the milk unit clusters was 60 s. The vacuum of the milking equipment (Flo-Star MAX, Boumatic Robotics GmbH, Kempten, Germany) was 45 kPa and the milk-to-rest ratio was at 60:40. After milking, the teats of the cows were dipped (Jod 5000, CID Lines N.V., Ieper, Belgium). A vaginal obstetrical follow-up examination was conducted to identify vaginal injuries or the presence of a second calf. All postpartum cattle received 150 mL of propylene glycol orally after milking. All multiparous cows received an oral calcium bolus (Bovikalc, Boehringer Ingelheim Vetmedica GmbH, Ingelheim am Rhein, Germany).

Cows in the CON group had no contact with their calves after removal from the maternity pen and were milked as described above. Cows in the OXY group had no contact with their calves after removal from the maternity pen, but were injected with 20 IU of oxytocin (Oxytocin ad us. Vet., aniMedica GmbH, Senden-Bösensell, Germany) i.m. (BMV injection syringe and MS Alu-Hub cannula 1.2 × 16 mm, MS Schippers GmbH, Kerken, Germany) into the neck region 3 min before manual

stimulation. Milking occurred as described above. For cows in the third group (CA), the calf was placed into a calf cart and located in front of the cow 3 min before manipulation of the cow and during the whole milking process.

Colostrum was harvested into a bucket weighing 3.48 kg. After each milking the bucket, including the colostrum, was weighed with a digital hanging scale (digital hanging scale, model no. XY-2003, Etekcity Corporation, Anaheim, CA, minimum weight 200 g, and maximum weight 50 kg) and then the weight of the bucket was subtracted. A colostrum sample (15 mL) was collected into a sterile plastic container (15 mL centrifuge tubes with screw caps, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and frozen (−20°C) until further analysis. Colostrum quality was assessed using a digital Brix refractometer (HI 96801, Hanna Instruments Deutschland GmbH, Vöhringen, Germany) by farm personnel and the first author. Frozen colostrum samples were stored on ice for transportation to laboratory of the Clinic of Animal Reproduction. Time between sampling of cows and pick-up to laboratory delivery never exceeded 7 d.

3.4.4 Colostrum Sample Analysis

For further assessment, the colostrum samples were thawed at ambient temperature until reaching 21°C, vortexed for 10 s, 1 mL aliquot was transferred to a sterile vial (Cryovial 2 mL; Simport, Bernard-Pilon, Germany) and shipped on ice to the Veterinary Science Department, Faculty of Veterinary Medicine, Ludwig Maximilian University of Munich, for IgG analysis.

The IgG in the colostrum was measured using a sandwich ELISA according to Erhard et al. (1999). Samples were diluted with PBS-Tween in a ratio of 1:50,000. The detection of the IgG concentration was based on coating and conjugating the IgG with anti-bovine IgG coupled to a peroxidase enzyme, which catalyzed a color change proportional to the IgG concentration of the sample. This color change was measured photometrically. Rabbit serum with anti-bovine IgG (5 µg/mL; A 5645, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was pipetted into each well of a 96-well polystyrene plate (F96 Cert. Maxisorp Nunc-Immuno Plate, Thermo Scientific GmbH, Darmstadt, Germany) and blocked with a 0.5% gelatin PBS solution. The diluted colostrum samples were inoculated into the uppermost cavity of each column. In addition, the samples were diluted by using a 2-logarithmic dilution series with PBS-Tween buffer in a ratio of 1:2. Furthermore, the peroxidase-linked rabbit anti-bovine IgG and the substrate solution (332 µL of stock solution, 10 mL

of Tetramethylbenzidine buffer, 30% H_2O_2) were added, which started the enzyme reaction. By applying 50 µL of 1 molar sulfuric acid, the reaction process was stopped. The photometric intensity was measured at 450 nm with an ELISA Reader (GENios, Tecan Germany GmbH, Crailsheim, Germany). The mean value of the IgG concentration in each well of one column resulted in the final colostrum concentration (mg of IgG/mL). Colostrum containing ≥ 50 mg/mL of IgG was regarded as high quality colostrum (McGuirk and Collins, 2004).

3.4.5 Data Collection and Statistical Analyses

Relevant cow data such as cow ID, parity, gestation length, days in the prepartum pen, calving ease, date and time of parturition, calf birth weight, sex of calf, twin births, and postpartum diseases (e.g., milk fever, retained fetal membranes, metritis) were obtained through the on-farm computer software (DairyCOMP 305, Valley Ag Software, Tulare, CA) and transferred to Microsoft Excel (Office 2013, Microsoft Deutschland Ltd.). Statistical analyses were performed using SPSS for Windows (version 22.0, SPSS Inc., IBM, Ehningen, Germany).

A priori sample size was calculated using MedCalc software (version 15.6.1, MedCalc, Mariakerke, Belgium). Based

on historical data from the farm regarding colostrum quantity (5.8 ± 2.9 kg), the sample size was calculated to detect an increase in colostrum quantity of 1.0 kg, assuming 80% power and a confidence level of 95%. Therefore, a total of 133 dairy cows per group were needed.

To evaluate the effect of 2 different treatment procedures to increase colostrum quantity and quality, 2 separate generalized linear mixed models were constructed using the GENLINMIXED procedure of SPSS. The outcome variable was either colostrum quantity (kg) or quality (mg of IgG/mL or % Brix). Cow was the experimental unit. According to the model-building strategies described by Dohoo et al. (2009), each parameter considered for the mixed model was separately analyzed in an univariate model, including the parameter as a fixed factor (i.e., categorical parameter) or covariate (i.e., continuous parameter). Only parameters resulting in univariate models with $P \leq 0.20$ were included in the final mixed model. Selection of the model that best fit the data was performed by testing each effect separately in a multivariate model and finding the model with the lowest value for the Akaike information criterion using a backward elimination procedure that removed all variables with $P > 0.10$ from the model. Regardless of the significance level, the intervention procedure was forced to remain in the model.

The initial model for colostrum quantity contained the following explanatory variables as fixed effects: treatment procedure (CON, OXY, and CA), parity (1, 2, and 3+), calving ease (score 1 to 4), employee responsible for calving and milking (1 to 8), calving time (morning from 06:00 to 14:00 h, afternoon from 14:00 to 22:00 h, night from 22:00 to 06:00 h), calving time during the day (1 to 24 h), calving day of the week (Monday until Sunday), harvesting time after calving (hours; continuous), calf sex (male vs. female), calf birth weight (continuous), gestation length (continuous), and days in the prepartum pen (continuous).

The initial model for colostrum quality contained the same explanatory variables as for colostrum quantity. Additionally, colostrum quantity (continuous) was included. We tested all biologically plausible 2-way interactions.

Correlation coefficients, minima, and maxima were determined using Excel (Office 2010, Microsoft Deutschland Ltd.).

3.5 Results

Overall, 567 animals were enrolled. Forty-six animals were excluded due to multiple reasons such as lameness (n = 1), bloody colostrum (n = 20), mastitic colostrum (n = 1), gestation length of less than 265 d (n = 7), twin births (n = 11), and missing

data (n = 6). For the final analyses, 521 animals (365 multiparous cows and 156 first-calf heifers) were considered regarding colostrum quantity (Figure 1). The mean harvesting time after calving was 47 ± 0.33 min with a range of minimum 5 min up to maximum of 4 h and 50 min after calving.

3.5.1 Colostrum Quantity

Information on colostrum quantity was available for 177, 163, and 181 animals in the CON, OXY, and CA group, respectively (Figure 1). No differences were observed in parity ($P = 0.82$) or calf birth weight ($P = 0.65$) among the 3 groups. The correlation coefficient of gestation length and calf birth weight was r = 0.49. The harvesting time after calving had no significant effect on colostrum quantity ($P = 0.33$). The mean colostrum quantity was 4.17 ± 0.30 kg (minimum: 0.00 kg, maximum: 28.40 kg). The treatment procedures had no effect on colostrum quantity ($P = 0.50$; Table 1; Figure 2). Parity ($P = 0.01$), calf birth weight ($P < 0.01$; Figure 2), and calving time ($P = 0.08$) affected colostrum quantity. Parity 2 cows had the lowest quantity of colostrum (3.74 ± 0.37 kg) compared with cows in parity 1 (4.75 ± 0.34 kg) and cows in parity 3 or greater (4.75 ± 0.38 kg). Cows calving during the night (4.93 ± 0.37 kg) had the highest

quantity of colostrum compared with cows calving in the morning (4.17 ± 0.38 kg) or afternoon (4.14 ± 0.34 kg; Table 1).

3.5.2 Colostrum Quality Measured by Sandwich ELISA

Information on colostrum quality was available for 165, 149, and 165 animals in the CON, OXY, and CA group, respectively (Figure 1). Forty-two colostrum samples could not be assessed with sandwich ELISA due to their high viscosity (12 samples in CON, 14 in OXY, and 16 in CA). There was no difference in parity ($P = 0.95$) and calf birth weight ($P = 0.51$) among the 3 groups. The mean IgG concentration was 54.6 ± 2.80 mg of IgG/mL (minimum: 14.5 mg of IgG/mL, maximum: 146.3 mg of IgG/mL). Colostrum quality was affected by the treatment procedures ($P = 0.04$; Figure 3). In addition, colostrum quantity ($P < 0.01$), parity ($P < 0.01$; Table 2; Figure 3), calving time ($P < 0.01$), harvesting time after calving ($P = 0.03$), and calving day ($P = 0.06$) had an effect on the IgG concentration in colostrum. Both treatment procedures [i.e., OXY with mean IgG concentration results of 57.0 mg of IgG/mL ($P = 0.02$) and CA with 56.0 mg of IgG/mL ($P = 0.04$)] resulted in higher IgG concentrations in colostrum compared with CON (50.7 mg of IgG/mL). With increasing colostrum quantity, the IgG concentration in colostrum (mg/mL) decreased in primiparous

cows (r = −0.21) and in multiparous cows (r = −0.13; Table 2). A longer time lag between calving and milking negatively affected the IgG concentration in colostrum (mg/mL). Concentration of IgG was higher for cows in parity 3 or greater (64.6 ± 2.59 mg of IgG/mL) compared with cows in parity 1 (48.5 ± 2.86 mg of IgG/mL) and cows in parity 2 (50.7 ± 2.89 mg of IgG/mL). Cows calving during the night had greater IgG concentrations (60.4 ± 2.92 mg of IgG/mL) compared with cows calving in the morning (51.9 ± 2.98 mg of IgG/mL) or afternoon (51.3 ± 2.71 mg of IgG/mL). Harvesting colostrum on quieter days, such as Sundays, resulted in higher IgG concentrations (61.4 ± 3.70 mg of IgG/mL).

3.5.3 Colostrum Quality Measured by Brix Refractometry

Information on colostrum quality measured by Brix refractometry was available for 175, 155, and 170 animals in the CON, OXY, and CA group, respectively (Figure 1). Twenty-one colostrum samples could not be assessed with Brix refractometer due to their high viscosity (2 samples in CON, 8 in OXY, and 11 in CA). The harvesting time after calving had no significant effect on colostrum quality (P = 0.73). There was no difference in parity (P = 0.76) and no difference in calf birth weight (P = 0.49) among the 3 groups. The mean result was 26.0 ± 0.20% Brix (minimum:

15.7% Brix, maximum: 39.7% Brix). Treatment procedures had no effect on colostrum quality ($P = 0.44$; Table 3; Figure 3). Colostrum quantity ($P < 0.01$; Table 3) and parity ($P < 0.01$; Table 3; Figure 3) affected colostrum quality. A negative association was observed between colostrum quantity and quality determined by Brix refractometry. With increasing colostrum quantity, the Brix readings (% Brix) decreased. The correlation coefficient was $r = -0.09$ in primiparous cows and $r = -0.17$ in multiparous cows. Brix readings were greater for cows in parity 3 or higher (27.7 ± 0.26% Brix) compared with cows in parity 1 (25.3 ± 0.30% Brix) and cows in parity 2 (25.0 ± 0.32% Brix). The correlation coefficient between the analysis with sandwich ELISA and the assessment via Brix refractometry was $r = 0.44$.

Table 1. Estimated effect of two treatment procedures at the first milking after calving aiming at increasing colostrum quantity (kg) in 521 Holstein dairy cows.

Variable	Estimate colostrum (kg)	SE[1]	95% CI		*P*-value
			Lower CI	Upper CI	
Intercept	-3.595	1.20	-5.953	-1.237	0.003
Parity					
Parity 1	Referent				
Parity 2	-1.007	0.41	-1.806	-0.207	0.014
Parity 3+	0.008	0.39	-0.758	0.775	0.983
Treatment procedure[2]					
Control group[3]	Referent				
Oxytocin group[4]	0.359	0.36	-0.342	1.060	0.314
Presence of the calf[5]	0.354	0.35	-0.327	1.035	0.308
Calving time					
Morning (6 a.m. – 2 p.m.)	Referent				
Afternoon (2 p.m. – 10 p.m.)	-0.025	0.38	-0.774	0.723	0.947
Night (10 p.m. – 6 a.m.)	0.769	0.42	-0.053	1.591	0.067
Calf birth weight	0.189	0.03	0.132	0.247	0.001

[1]SE = Standard error of the estimate.

[2]Different treatment procedures at the first milking after calving aiming at increasing colostrum quantity (kg) in 521 Holstein dairy cows.

[3]Control group (CON; n = 177). Cows in the control group had no contact to their calves after calving.

[4]Oxytocin group (OXY; n = 163). Cows in the oxytocin group had no contact to their calves and were injected with 20 IU i.m. oxytocin 3 min before first milking.

[5]Presence of the calf group (CA; n = 181). The calf was placed into a calf cart and located in front of the cow 3 min before manipulation of the cow and during the whole milking process.

Table 2. Estimated effect of two treatment procedures at the first milking after calving aiming at increasing colostrum quality (mg IgG/mL) in 479 Holstein dairy cows.

Variable	Estimate colostrum (mg IgG/mL)	SE[1]	95% CI		*P*-value
			Lower CI	Upper CI	
Intercept	55.440	4.73	46.140	64.740	0.001
Parity					
Parity 1	Referent				
Parity 2	2.073	2.89	-3.603	7.748	0.473
Parity 3+	16.069	2.59	10.976	21.163	0.001
Treatment procedure[2]					
Control group[3]	Referent				
Oxytocin group[4]	6.351	2.73	0.986	11.716	0.020
Presence of the calf[5]	5.300	2.61	0.165	10.435	0.043
Colostrum quantity	-1.658	0.32	-2.280	-1.035	0.001
Calving time					
Morning (6 a.m. – 2 p.m.)	Referent				
Afternoon (2 p.m. – 10 p.m.)	-0.644	2.88	-6.294	5.007	0.823
Night (10 p.m. – 6 a.m.)	8.469	3.18	2.214	14.725	0.008
Harvesting time (hours)	-3.213	1.43	-6.011	-0.414	0.025
Calving day of the week					
Monday	Referent				
Tuesday	-5.581	3.93	-13.312	2.150	0.157
Wednesday	-6.499	4.01	-14.378	1.381	0.106
Thursday	-4.884	3.88	-12.504	2.735	0.208
Friday	-0.994	4.16	-9.166	7.178	0.811
Saturday	-7.850	3.96	-15.623	-0.076	0.048
Sunday	3.714	4.16	-4.464	11.892	0.373

[1]SE = Standard error of the estimate.

[2]Different treatment procedures at the first milking after calving aiming at increasing colostrum quality (mg IgG/mL).

[3]Control group (CON; n = 165). Cows in the control group had no contact to their calves after calving.

[4]Oxytocin group (OXY; n = 149). Cows in the oxytocin group had no contact to their calves and were injected with 20 IU i.m. oxytocin 3 min before first milking.

[5]Presence of the calf group (CA; n = 165). The calf was placed into a calf cart and located in front of the cow 3 min before manipulation of the cow and during the whole milking process.

Table 3. Estimated effect of two treatment procedures at the first milking after calving to stimulate oxytocin secretion aiming at increasing colostrum quality (% Brix) in 500 Holstein dairy cows.

Variable	Estimate colostrum (% Brix)	SE[1]	95% CI		*P*-value
			Lower CI	Upper CI	
Intercept	26.336	0.41	25.530	27.140	0.001
Parity					
Parity 1	Referent				
Parity 2	-0.279	0.40	-1.061	0.504	0.485
Parity 3+	2.490	0.36	1.788	3.192	0.001
Treatment procedure[2]					
Control group[3]	Referent				
Oxytocin group[4]	-0.343	0.37	-1.069	0.383	0.354
Presence of the calf[5]	0.116	0.36	-0.590	0.823	0.746
Colostrum quantity	-0.215	0.04	-0.302	-0.128	0.001

[1]SE = Standard error of the estimate.

[2]Different treatment procedures at the first milking after calving aiming at increasing colostrum quality (% Brix)

[3]Control group (CON; n = 175). Cows in the control group had no contact to their calves after calving.

[4]Oxytocin group (OXY; n = 155). Cows in the oxytocin group had no contact to their calves and were injected with 20 IU i.m. oxytocin 3 min before first milking.

[5]Presence of the calf group (CA; n = 170). The calf was placed into a calf cart and located in front of the cow 3 min before manipulation of the cow and during the whole milking process.

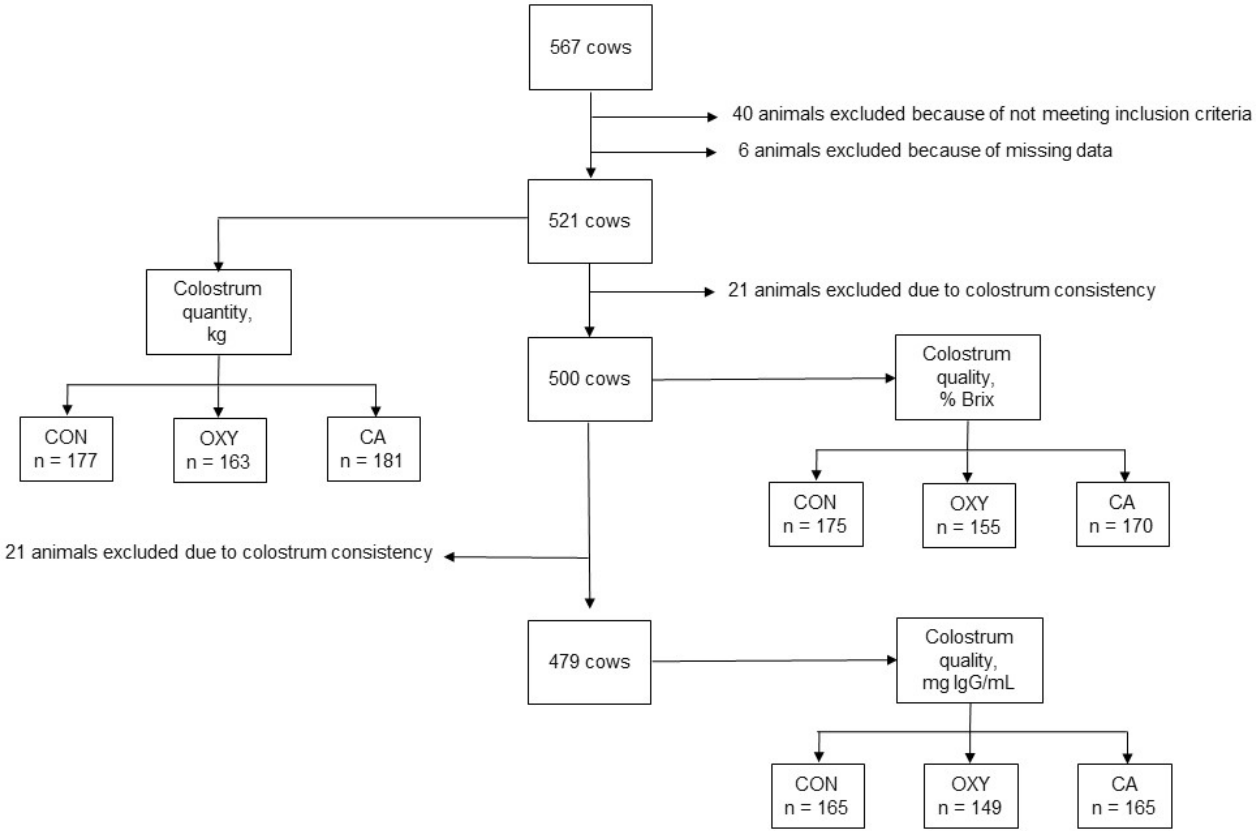
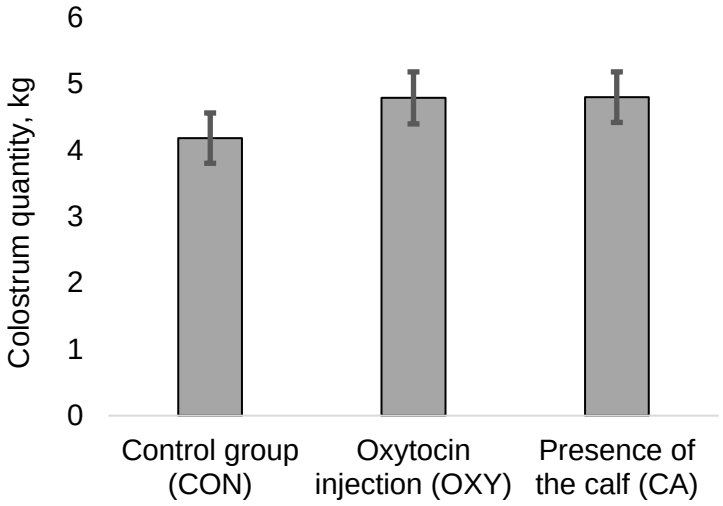
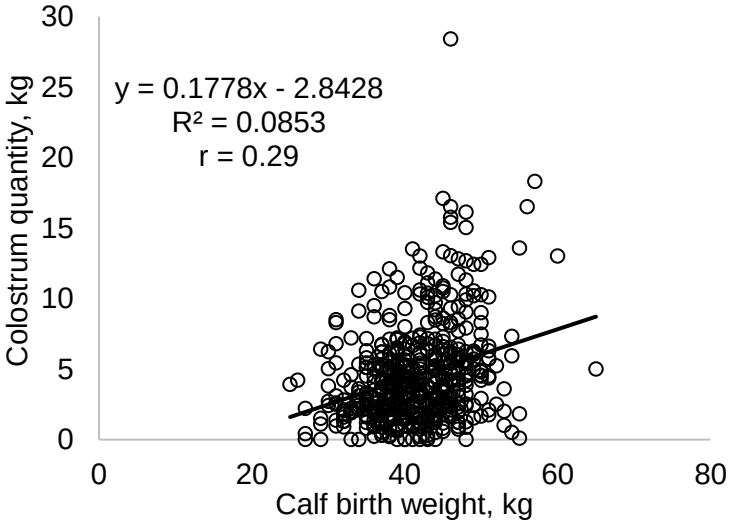

567 cows
40 animals excluded because of not meeting inclusion criteria
6 animals excluded because of missing data
521 cows
Colostrum quantity, kg
21 animals excluded due to colostrum consistency
500 cows
Colostrum quality, % Brix
CON n = 177
OXY n = 163
CA n = 181
CON n = 175
OXY n = 155
CA n = 170
21 animals excluded due to colostrum consistency
479 cows
Colostrum quality, mg IgG/mL
CON n = 165
OXY n = 149
CA n = 165

Figure 1. Flowchart of total number of animals enrolled in the study and considered for final analyses. Number of animals included in each analysis might differ due to several reasons (e.g., inclusion criteria were not met, missing data about colostrum quantity and quality, colostrum was too viscous to be considered for sandwich ELISA analysis or Brix refractometry). Cows in the control group (CON) had no contact with their calves after calving. Cows in the oxytocin group (OXY) had no contact with their calves and were injected with 20 IU of oxytocin i.m. 3 min before first milking. In the presence of the calf group (CA), the calf was placed into a calf cart and located in front of the cow 3 min before manipulation of the cow and during the whole milking process.

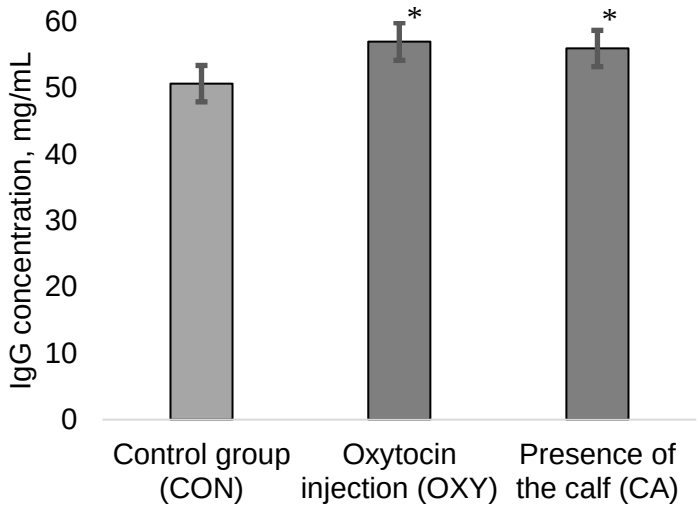

Colostrum quantity, kg
6
5
4
3
2
1
0
Control group (CON)
Oxytocin injection (OXY)
Presence of the calf (CA)

a)

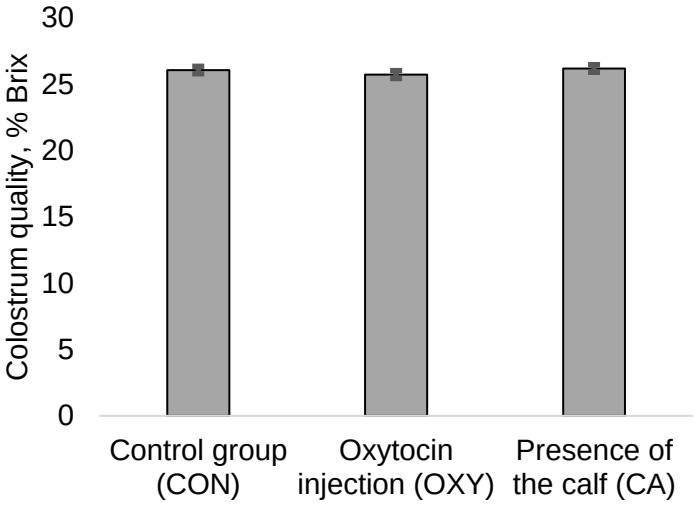

Colostrum quantity, kg
y = 0.1778x - 2.8428
R² = 0.0853
r = 0.29
30
25
20
15
10
5
0
0
20
40
60
80
Calf birth weight, kg

b)

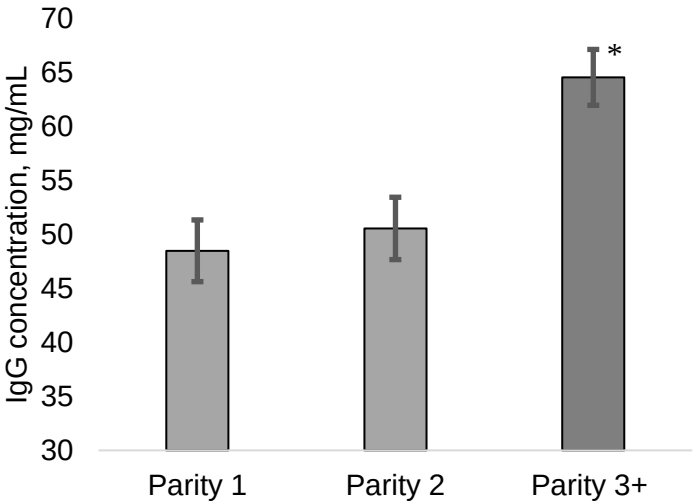
Figure 2. a) Effect of treatment procedures at harvesting of colostrum on colostrum quantity (kg, means ± SE). Cows in the control group (CON; n = 177) had no contact with their calves. Cows in the oxytocin group (OXY; n = 163) had no contact with their calves and were injected with oxytocin (20 IU i.m.) 3 min before first milking. For cows in the presence of the calf group (CA; n = 181), the calf was placed into a calf cart and located in front of the cow 3 min before manipulation of the cow and while milking. **b)** Effect of calf weight on colostrum quantity.


IgG concentration, mg/mL
60
50
40
30
20
10
0
*
*
Control group (CON)
Oxytocin injection (OXY)
Presence of the calf (CA)

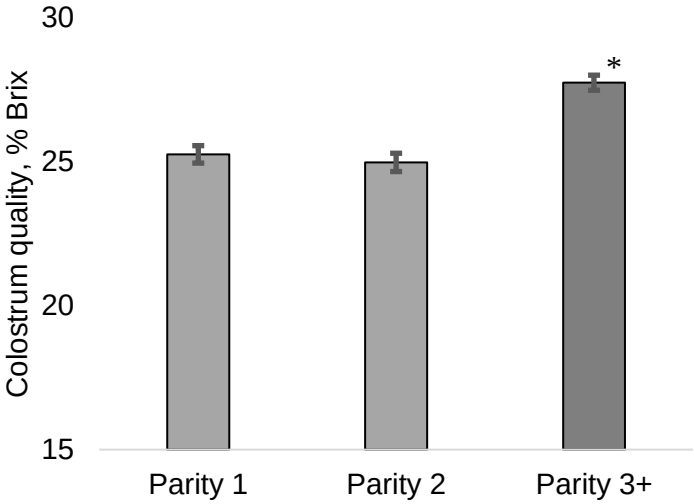
a)


Colostrum quality, % Brix
30
25
20
15
10
5
0
Control group (CON)
Oxytocin injection (OXY)
Presence of the calf (CA)

b)


70
65
60
55
50
45
40
35
30
IgG concentration, mg/mL
*
Parity 1
Parity 2
Parity 3+

c)


30
25
20
15
Colostrum quality, % Brix
*
Parity 1
Parity 2
Parity 3+

d)

Figure 3. a) and **b)** Effect of different treatment procedures at harvesting of colostrum on colostral IgG concentration (mg/mL, means ± SE) and on colostrum quality in % Brix (% Brix, means ± SE). Different treatment procedures before the first milking have been evaluated to improve harvesting of high quality colostrum. Cows in the control group (CON) had no contact with their calves. Cows in the oxytocin group (OXY) had no contact with their calves and were injected with oxytocin (20 IU i.m.) 3 min before first milking. For cows in the presence of the calf group (CA), the calf was placed into a calf cart and located in front of the cow 3 min before manipulation of the cow and while milking [animals enrolled: a) CON, n = 165; OXY, n = 149; CA, n = 165; b) CON, n = 175; OXY, n = 155; CA, n = 170]. **c)** and **d)** Effect of parity at harvesting of colostrum on colostral IgG concentration (mg/mL, means ± SE) and on colostrum quality in % Brix (% Brix, means ± SE). Different treatment procedures at harvesting the first colostrum have been evaluated to increase colostrum quality. Parity was one of different parameters that were taken into consideration [animals enrolled: c) cows in parity 1, n = 144; parity 2, n = 130; parity 3 or greater, n = 205; d) cows in parity 1, n = 149; parity 2, n = 133; parity 3 or greater, n = 218].

3.6 Discussion

The main findings of the present study were (1) the treatment procedure did not affect colostrum quantity; (2) administration of oxytocin or the presence of the calf increased IgG concentration in colostrum of cows compared with the control group; (3) parity was associated with colostral IgG concentration; and (4) calf birth weight, calving time, and calving day were positively associated with colostrum quantity. Hence the treatment procedure had no overall effect on colostrum quantity; both treatments were beneficial for IgG concentration in colostrum.

3.6.1 Colostrum Quantity

There was a negative association of colostrum quantity and quality regardless of the measurement (i.e., sandwich ELISA or Brix refractometry). This is in line with an older study that also reported a negative correlation between colostrum quantity and quality with a correlation coefficient of $r = -0.29$ (Pritchett et al., 1991). This effect is most likely due to dilution of colostral IgG. The increasing colostrum quantity is based on water diffusion, because of a higher secretion of lactose into the udder when the lactation begins, whereas the absolute amount of IgG remains the same (Baumrucker et al., 2010; Morin et al., 2010).

3.6.2 Colostrum Quality

Forty-eight percent of the colostrum samples contained ≥ 50 mg of IgG/mL and 6.5% contained ≥ 100 mg of IgG/mL. The mean IgG concentration in the present study was 54.6 ± 2.80 mg of IgG/mL with a range of 14.5 to 146.3 mg of IgG/mL. This is in agreement with a previous study (Baumrucker et al., 2010) in which a range of 9 to 166 mg of IgG/mL was reported.

For the comparison of IgG concentration across studies, it is important to consider the analytical methods used. The common techniques were radial immunodiffusion (RID; Kehoe et al., 2011; Rivero et al., 2012) and ELISA (Baumrucker et al., 2010; Nowak et al., 2012). In addition, preparation of colostrum samples varied between studies. Baumrucker et al. (2010) removed the colostral fat before analysis with ELISA, whereas others did not (Morrill et al., 2012). It has been established that removal of colostral fat before the analysis can cause overestimation of IgG concentration using RID (Fleenor and Stott, 1981). Such methodological differences create challenges when comparing results across studies.

Both treatment procedures (OXY and CA) were beneficial for colostrum quality. We speculate that both procedures led to a high concentration of oxytocin in the blood, which might have affected the integrity of the mammary tight junctions and presumably led to a higher IgG transfer into the udder (Stelwagen

and Singh, 2014). According to Stelwagen and Singh (2014) the tight junction integrity of the mammary gland might be compromised following administration of high, nonphysiological, doses of exogenous oxytocin. The increased permeability is likely due to a disruption of the cell-cell contact as a result of the mechanical forces caused by the sudden alveolar contraction (Stelwagen and Singh, 2014).

Another explanation for higher IgG concentrations in OXY and CA might be that the high concentration of exogenous or endogenous oxytocin leads to the removal of residual milk from the udder, which might possibly result in higher IgG concentrations in colostrum. About 15 to 20% residual milk stays in the udder after milking. Nostrand et al. (1991) found that the use of exogenous oxytocin increased lactation milk production during the declining phase of lactation after peak milk yield. However, mean fat and protein percentages did not differ for oxytocin and control cows during lactation (Nostrand et al., 1991). We could not detect a difference in colostrum quantity for cows receiving exogenous oxytocin (OXY) and presumably a higher endogenous oxytocin concentration (CA) compared with CON cows.

In previous studies, the correlation between RID and digital refractometry ranged from 0.64 to 0.87 (Vandeputte et al., 2014; Bartier et al., 2015; Coleman et al., 2015; Morrill et al.,

2015). According to Bielmann et al. (2010), a digital refractometer can determine the IgG content in colostrum measured by RID with a correlation of r = 0.71 and acceptable test sensitivities and specificities. In our study there was only a weak correlation between sandwich ELISA and Brix refractometry (r = 0.44). Obviously, results obtained by sandwich ELISA and RID cannot be directly compared (Dunn et al., 2017). Therefore, the results in our study generated by sandwich ELISA limits the comparability with other studies using RID. It remains speculative why there was only poor agreement between the 2 methods. According to Elsohaby et al. (2017), variations in the correlation coefficients could be related to the different non-IgG components in colostrum. The measurement via Brix refractometry assesses IgG concentrations indirectly, through assessing total dissolved solids that affect the sucrose concentration. These are affected by dry period length (Rastani et al., 2005), vaccination status of the dam (Hodgins and Shewen, 1996), and season of calving (Morin et al., 2001). Further research is needed to compare the different analytical methods.

3.6.3 Parity

According to Gulliksen et al. (2008), the concentration of colostral IgG increases with increasing parity until reaching the

fourth lactation. Older cows seemed to produce colostrum with higher IgG concentrations, being exposed to antigens for a longer time during their life than younger cows. Antibodies are transferred from serum into colostrum and the colostral IgG concentration increases with the number of lactations. Consequently, the parity itself had a positive influence on colostrum quality (Conneely et al., 2013). In our study, primiparous cows and cows in 2nd parity had lower colostrum quality determined by both methods compared with older cows, which is consistent with previous studies (Pritchett et al., 1991; Gulliksen et al., 2008). Van Saun and Sniffen (2014) recommended prepartum feeding diets with at least > 1,100 g/d, with 1,300 g/d of MP being better amount. The feeding diet in the present study was slightly above 1,100 g/d (1,189 g/d), but below the 1,300 g/d recommendation, which might explain the weak percentage (48%) of high quality colostrum found. Furthermore, it might explain the poor performance of 2nd parity cows. As 41% of colostral samples drawn from primiparous cows exceeded the cutpoint of 50 mg of IgG/mL, it is worthwhile to measure colostrum quality on farm and discard only the low quality colostrum.

3.6.4 Calf Birth Weight and Gestation Length

We observed a positive association between calf birth weight and colostrum quantity. Gestation length and calf birth weight may be positively correlated with colostrum quantity or related to the dam's BW. Gestation length and calf birth weight are related, since the growth of the fetus in the last trimester of gestation increases considerably (Van Saun and Sniffen, 2014). In the present study, the correlation coefficient of gestation length and calf birth weight was r = 0.49. According to Karl and Staufenbiel (2016), the average IgG concentration in colostrum depends on gestation length. Cows up to 275 d gestation length had a colostral IgG concentration of 51.8 mg/mL, whereas cows with 276 to 285 d had 25.6 mg/mL and cows with more than 285 d had 26.2 mg/mL (Karl and Staufenbiel, 2016). The average IgG concentration in our study was 54.4 mg/mL for cows with a gestation length of 265 up to 275 d (n = 191), 55.6 mg/mL for cows with 276 to 285 gestation days (n = 273), and 49.3 mg/mL for cows with 286 to 289 gestation days (n = 34).

Furthermore, BW of the dam is associated with calf weight at birth (Berry et al., 2004). As BW is also associated with milk quantity, it might also affect colostrum quantity (Berry et al., 2004; Conneely et al., 2013). While BW of the cows was not measured in this study, it might be an explanation for this association.

In addition, bovine placental lactogen, a hormone produced by the syncytiotrophoblasts of the placenta, correlates with calf birth weight and milk yield in singleton cows (Patel et al., 1996). A stronger secretion capacity of bovine placental lactogen in certain cows could affect calf birth weight and colostrum quantity, but the reason for this difference remains speculative.

3.6.5 Calving Time, Harvesting Time, and Day of Calving

A positive association between calving time and colostrum quantity, as well as IgG concentration in colostrum, was detected. Cows calving during the night had the highest quantity and IgG concentration of colostrum compared with cows calving in the morning or afternoon. We speculate that our results were the outcome of lower stress levels of the cows calving at night. During the night, far fewer farm activities occurred and the noise level was significantly reduced. In addition, we observed higher IgG concentration in colostrum, if calving and harvesting of colostrum took place on Sundays. Again, we speculate that lower stress levels of cows calving on Sundays could be the possible explanation for this observation. This finding needs to be validated with a multicentric study design.

In agreement with previous studies (Moore et al., 2005; Morin et al., 2010), the IgG concentration in colostrum was

negatively associated with the interval from calving to colostrum collection. A longer time lag between calving and milking reduced the IgG concentration in colostrum. According to Morin et al. (2010) the colostral IgG concentration decreases 3.7% per hour after calving.

The study was conducted during fall and winter with mean ambient temperatures of 9, 7, and 4°C in October, November, and December, respectively. The exposure of cattle to high ambient temperatures during late pregnancy has been associated in different studies with poorer colostrum composition, including lower mean concentrations of colostral IgG and IgA, and other components, such as total protein, casein, lactalbumin, fat, and lactose (Godden, 2008). The negative effects of heat stress may influence DMI, resulting in nutritional restriction. Furthermore, a reduced mammary blood flow causes impaired transfer of IgG and nutrients from the blood stream to the udder, or impaired immune reactivity of mammary gland plasmacytes that produce IgA (Godden, 2008; Tao and Dahl, 2013). Part of the included animals were exposed to higher ambient temperatures during late pregnancy. Resulting heat stress could have affected colostrum quality, which could explain the low percentage of high quality colostrum found (48%). However, this remains an assumption because heat stress parameters were not assessed in the present study.

3.7 Study limitations

The study was carried out on only one farm. According to Sargeant et al. (2010), a randomized trial conducted at a single site may not be representative of the variety of possible clinical situations. External validity in the present study is limited. Therefore, the results need to be validated with a multicentric study design. Also, the 3 treatment procedures were not randomly allocated on a cow basis but on a daily basis. This approach was chosen to increase compliance with farm employees. We are aware that a random allocation of each cow to 1 of the 3 groups would have been more robust. Based on previous experience, however, we assumed that a study design with a random allocation on a cow basis would have reduced compliance significantly. Furthermore, in the present study 2 treatment procedures (OXY and CA) were compared with a CON group. The option of an i.m. application of saline solution as a placebo in the CON group was rejected, which is an obvious study limitation. A study design with a second control group considering the application of a placebo might have enhanced the comparison of the different treatment procedures.

3.8 Conclusions

Overall, none of the treatment procedures improved colostrum quantity. However, the administration of parental oxytocin and presence of the calf increased IgG concentration in colostrum of cows compared with the control group. The external validity in our study is limited; therefore, the results should be validated with a multicentric study design. Future studies should involve several farms to increase external validity and measure the biological effect of the treatments (i.e., assessing the serum total protein, the IgG concentration of calves, or the health effects by evaluating clinical signs of calves).

3.9 Acknowledgements

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4 ADDITIONAL UNPUBLISHED WORK

Evaluation of different analytical methods to assess failure of passive transfer in neonatal calves.

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4.1 Abstract

The objective of this study was to evaluate different analytical methods to assess failure of passive transfer (FPT) in neonatal calves. We hypothesized that three different media (i.e., centrifuged serum, centrifuged plasma, filtered plasma) and different analytical methods to assess FPT [i.e., ELISA, capillary electrophoresis (CE), Brix refractometer and handheld optical refractometer] were highly correlated with the gold standard radial immunodiffusion (RID) and would generate comparable results. Serum and plasma blood samples were collected from Holstein Friesian calves (n = 216) aged 1 to 7 days, from two commercial dairy herds in Northeast Germany.

The RID analysis showed 59 (27%) out of 216 calves had serum IgG concentrations < 10 mg/mL and 157 calves (73%) had ≥ 10 mg/mL, respectively. The mean IgG concentration (means ± SD) was 17.1 ± 9.8 mg/mL and the range was 0.8 to 47.8 mg IgG/mL. The correlation in serum between RID and CE was r = 0.97 and between RID and ELISA was r = 0.90, respectively. Furthermore, CE and ELISA were highly correlated (r = 0.89). Both refractometry methods were highly correlated with RID using either centrifuged serum, centrifuged plasma, or filtered plasma (Brix refractometer: r = 0.84; r = 0.80; r = 0.78; handheld optical refractometer: r = 0.83; r = 0.81; r = 0.80). Test

characteristics [optimum thresholds, sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV) and area under the curve (AUC)] for CE, ELISA, as well as the handheld optical and digital refractometer to identify calves with FPT using three different media were determined by ROC curve analyses by using RID as the reference value.

Optimal thresholds to assess FPT using plasma were higher compared with serum regardless of the method of plasma harvesting. The four different devices had comparable AUC irrespective of the medium used. In conclusion, all methods can be used for the assessment of FPT.

4.2 Key words

capillary electrophoresis, plasma filter, failure of passive transfer, neonatal calves.

4.3 Introduction

An adequate colostrum supply is beneficial for the neonatal calves' health (Cuttance et al., 2018; Lora et al., 2018). It positively affects the metabolism of the neonatal calf and has the potential to influence the lifetime performance as a dairy cow (Faber et al., 2005; Soberon et al., 2012; Soberon and Van Amburgh, 2013). During the first lactation, an inadequate

colostrum supply can be linked with decreased milk yield and increased culling rates (Chuck et al., 2018).

The timely delivery of high quality colostrum in an appropriate quantity is necessary for a high absorption rate of intestinal immunoglobulin G (**IgG**), which is essential for successful passive transfer in neonatal dairy calves (Weaver et al., 2000; Godden et al., 2009; Godden, 2017; Kertz et al., 2017). Sufficient maternal IgG supply via colostrum is crucial, since during gestation, the bovine epitheliochorial placenta impedes the transfer of IgG from the dam to the fetus. To acquire passive immunity, the neonatal calf depends on the absorption of maternal IgG from colostrum across the small intestinal epithelial cells before the cessation of intestinal transport 24 to 36 h after birth occurs (Weaver et al., 2000; Barrington and Parish, 2001). The absorption of maternal IgG is influenced by many factors, e.g., timing of the first feeding (Besser et al., 1985), volume of colostrum fed (Stott et al., 1979a), and quality of the colostrum (Pritchett et al., 1991). Good quality of colostrum is achieved with an IgG concentration ≥ 50 g/L, and total bacterial and fecal coliform counts should not exceed 100,000 and 10,000 cfu/mL (Weaver et al., 2000; McGuirk and Collins, 2004; Godden, 2008). Therefore, the absorption of maternal IgG also depends on the conditions from which the colostrum was collected and stored

(Weaver et al., 2000; Godden, 2008), on the apparent efficiency of absorption (**AEA**) (Stott et al., 1979c; Rajala and Castren, 1995), and on the abomasal emptying rate (Mokhber-Dezfooli et al., 2012).

Insufficient absorption of maternal IgG or failure of passive transfer (**FPT**) is defined by a serum IgG concentration below 10 mg/mL in neonatal calves aged 24 to 48 h (Weaver et al., 2000; Godden, 2008). In addition to serum samples, the use of different media, such as centrifuged plasma or filtered plasma are also appropriate for the assessment of FPT (da Costa Corrêa Oliveira et al., 2019; Elsohaby et al., 2019). The use of filtered plasma for the assessment of FPT offers the advantage that a large number of blood samples can be collected and analyzed without the need of centrifugation (da Costa Corrêa Oliveira et al., 2019). Therefore, the regular control of FPT can be well integrated into the daily work routine of a dairy farm. Different studies used either serum or plasma samples to assess FPT or a smaller number of samples to conduct the direct comparison of the two media (MacFarlane et al., 2014; Villarroel et al., 2014). Elsohaby et al. (2019) and da Costa Corrêa Oliveira et al. (2019) are the first studies to report a direct comparison between serum and plasma samples from the same dairy calves using a higher sample size (n = 217; n = 227).

Radial immunodiffusion (**RID**) is considered the gold standard in assessing the serum IgG concentration in neonatal calves (Bielmann et al., 2010). Laboratory-based methods, such RID and ELISA, have the disadvantage of being technically demanding, and require trained laboratory technicians (Davis and Giguère, 2005; Hogan et al., 2015). The measurement of serum total protein (**TP**) determined by refractometry is a simple alternative to indirectly assess the serum IgG concentration, TP represents the concentration of immunoglobulins (Calloway et al., 2002; Bielmann et al., 2010; Deelen et al., 2014; Buczinski et al., 2018). Refractometry can be performed with optical devices that measure TP concentration (g/dL) or digital Brix refractometers that evaluate total solids (**TS**) by using the refractive index in % Brix. Both refractometry methods are highly correlated with the IgG concentration determined by RID, if calves are tested between 24 hours and 7 days of age (McGuirk and Collins, 2004; Deelen et al., 2014; Buczinski et al., 2018). Thresholds used to identify calves with successful passive transfer range from 4.6 to 5.8 g/dL TP in serum (McCracken et al., 2017; Lee et al., 2008) and from 7.1 until 8.8% Brix, respectively (McCracken et al., 2017; Cuttance et al., 2017). According to McGuirk and Collins (2004), a successful passive transfer is acquired at ≥ 5.2 g/dL serum TP in healthy calves or

≥ 5.5 g/dL in ill calves. Using a herd-based testing for monitoring passive transfer, a minimum of 12 calves should be tested. An adequate colostrum management results in 80% of the calves having ≥ 5.5 g/dL serum TP (McGuirk and Collins, 2004). If more than 20% of the calves have lower serum TP levels, there is a risk for a herd FPT problem. Using a digital Brix refractometer for FPT assessment the cutpoint is at 8.4% Brix, respectively (Deelen et al., 2014).

Capillary electrophoresis (**CE**) is an analytical method based on the movement of charged particles in a liquid medium under the influence of an electric field. The migration velocities of different particles depend on their charge, shape, size, as well as on the solution environment and the strength of the electric field. Therefore, different particles are separated during the migration. In CE, this separation takes place in a capillary tube using electrolyte solution. In human medicine, CE and capillary gel electrophoresis (**CGE**) are well-established and common methods used for protein separation (Zhu et al., 2012). In contrast to CE, the medium for separation that is used for CGE is a gel (Bennett et al., 1994). In bovine veterinary medicine, CE and CGE are rarely used for protein and IgG analysis, although protein analysis by CGE has been successfully used to measure bovine IgG concentration (Bennett et al., 1994). Nevertheless,

CE or CGE has not been validated for assessment of FPT in neonatal calves.

The objective of this study was to evaluate different analytical methods to assess FPT in neonatal calves. We hypothesized that three different media (i.e., centrifuged serum, centrifuged plasma, filtered plasma) and different analytical methods to assess FPT (i.e., ELISA, CE, Brix refractometer and handheld optical refractometer) were highly correlated with the gold standard RID and generate comparable results.

4.4 Materials and Methods

4.4.1 Dairy Farm, Animal Enrollment and Sample Collection

The study was conducted from August to October 2018 on two commercial farms in Northeast Germany. Since all the required material was obtained during routine farm management practices to monitor FPT, the study was in accordance with the Institutional Animal Care and Use Committee of the Freie Universität Berlin. A sample size calculation was performed as described by Moinester and Gottfried (2014) for different half-widths (**w**) and Pearson correlation coefficients (**r**), based on CIxcorr, expecting the desired 95% CI. Clxcorr is a R function, which determines CI for given Pearson correlation coefficients

calculating lower and upper levels, widths for CI, significance α and sample size n (Moinester and Gottfried, 2014). For r = 0.80 and w = 0.05 a sample size requirement of n = 205 is needed.

Clinically healthy Holstein Friesian calves (n = 216) were enrolled in the study between 24 h and 7 d of age. The new intake of calves took place on a weekly basis and the relevant data, such as date and time of parturition, sex of the calf, twin births, colostrum uptake and the identification number were obtained from the on-farm documentation. Calves were considered clinically healthy, if no visible signs of disease were observed. Calves suffering from diarrhea, pneumonia or dehydration were rejected from the study. Diarrhea was defined as visibly loose feces of decreased consistency. Pneumonia was defined as visibly detectable tachypnea with nasal discharge (serous, mucous or purulent) and dehydration was defined with reduced skin turgor.

Whole blood of calves was collected by jugular vein puncture using a 20-gauge, 1.0-inch needle (Vacuette 20 x 1 sterile cannula, Greiner Bio-One GmbH, Kremesmünster, Austria) and two sterile, plastic, vacutainer tubes. A vacutainer tube without anticoagulant (9 mL, Vacuette CAT Serum, Greiner Bio-One GmbH) and a lithium-heparin tube (9 mL, Vacuette LH, Greiner Bio-One GmbH) were used. Serum samples were

centrifuged at 4.500 × *g* for 6 min at approximately 20°C on the farm 1 to 2 h after collection and were directly assessed via a handheld optical refractometer (RF.5612 Handheld refractometer, Euromex Holland, Arnhem, Netherlands) and a digital Brix refractometer (Misco PA201, MISCO, Solon OH, USA). Plasma filtration and plasma centrifugation was carried out at approximately 20°C in the laboratory of the Clinic for Animal Reproduction, Faculty of Veterinary Medicine, Freie Universität Berlin, 6 h after sample collection. For the transportation to the Freie Universität Berlin, all samples were stored on ice.

Filtered plasma obtained through a disposable filter had been validated as an analytical medium for FPT assessment in dairy calves using refractometry (da Costa Corrêa Oliveira et al., 2019). Before plasma filtration, every lithium-heparin sample was swiveled with a roll mixer for blood samples (Roll mixer RS-TR 5, Phoenix instrument, Garbsen, Germany) for 10 min. With a disposable syringe (5 mL HS Luer sterile disposable syringe, Henry Schein, Langen, Germany), 2 mL of lithium-heparin blood was aspirated and injected into the inlet reservoir of a plasma filter (2-Drop-Filter, Pharmadoc, Lübeck, Germany), which generated two drops of plasma. For each measurement, a new filter was used. Afterwards, the lithium-heparin blood samples were centrifuged at 4.500 × *g* for 6 min to obtain plasma.

For the evaluation of FPT all the different samples, i.e. centrifuged serum, filtered and centrifuged plasma were measured by a digital Brix refractometer in % Brix (Misco PA201, MISCO) and by a handheld optical refractometer (RF.5612 Handheld refractometer, Euromex Holland) assessing TP in g/dL. Both refractometers are optical devices built to analyze the concentration of water-soluble solids measuring the refractive index. Furthermore, the digital Brix refractometer assesses the rapidity at which the emitted beam of light passes through the fluid. At high density, the light passes more slowly through the fluid. Both devices have automatic temperature calibration so that accurate measurements are possible between 0 and 50°C (Misco PA201), and 0 and 30°C (Handheld refractometer). Before each batch of samples, the devices were calibrated with distilled water at room temperature (20°C).

Four aliquots of serum and one aliquot of centrifuged plasma were collected for further analysis. One and a half mL of serum or plasma were pipetted into a sterile vial (Cryovial 2 mL, Simport, Bernard-Pilon, Germany) to obtain the aliquots. One serum aliquot was sent frozen to the Department of Veterinary Science, Chair of Animal Welfare, Ethology, Animal Hygiene and Animal Husbandry (Faculty of Veterinary Medicine, LMU Munich, Germany) for IgG testing via sandwich ELISA according to

Erhard et al. (1999). Furthermore, one serum aliquot was sent to an accredited commercial laboratory (Laboklin, Bad Kissingen, Germany; accreditation number: DIN EN ISO IEC 17025:2005) to assess the IgG concentration via CE. Another aliquot was sent to The Saskatoon Colostrum Co. Ltd. (SCCL), in Canada for IgG assessment using single RID.

4.4.2 Serum and Plasma Sample Analyses

At the Department of Veterinary Science in Munich, the IgG analysis was assessed by sandwich ELISA. The method was based on coating and conjugating rabbit anti-bovine IgG coupled to a peroxidase enzyme (A 5295, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) with the serum IgG of the calves. Proportional to the IgG concentration of the sample, the peroxidase enzyme induced a color change, which could be quantified photometrically at 450 nm using an ELISA Reader (GENios, Tecan Germany GmbH, Crailsheim, Germany).

The serum samples were diluted with PBS-Tween in a ratio of 1:10,000. Rabbit serum with anti-bovine IgG (5 µg/mL) (A 5645, Sigma-Aldrich Chemie GmbH) was pipetted into each well of a 96-well polystyrene plate (F96 Cert. Maxisorp Nunc-Immuno Plate, Thermo Scientific GmbH, Darmstadt, Germany) and blocked with a 0.5% gelatin PBS solution. The serum samples

were inserted at the uppermost well of each column and diluted by using a two-logarithmic dilution series with PBS-Tween buffer. Thereafter, the peroxidase-linked rabbit anti-bovine IgG was pipetted into each well and later the substrate solution [332 µL stock solution, 10 mL 3,3′,5,5′-tetramethylbenzidine-buffer (**TMB**) and 30% H_2O_2] was added. The enzyme reaction took place. Fifty µL of 1 *M* sulphuric acid was applied, whereby the reaction process was stopped and the photometric intensity was measured. The final IgG concentration (mg/mL) of each serum sample was determined by the mean value of each column.

For CE analysis, the serum samples were diluted with NaCl 0.9% (B. Braun Melsungen AG, Melsungen, Germany) in a ratio of 1:2 and analyzed via CE. Silica capillaries were the main component of the CE apparatus (Minicap Immunotyping, Sebia SA, Lisses, France). In these silica capillaries, serum proteins were separated by their electrophoretic mobility and electroosmotic flow at high voltage on the anode or cathode in an alkaline buffer. The use of quartz capillaries enabled UV detection, whereby proteins were directly detected during migration by UV absorbance. This generates different concentration peaks that represent the concentration of the various protein components. The γ-globulin peak is clearly separable from other peaks and it consists mainly of IgG. The

relative IgG concentration obtained by CE was recalculated with the total protein concentration determined by a modular analyzer (Cobas c701 module, Roche Diagnostics Deutschland GmbH, Mannheim, Germany). This method has been validated internally in the accredited commercial laboratory (Laboklin) with an intra assay coefficient of variation of < 6.4%, and an inter assay coefficient of variation of < 6.3%, respectively.

Radial immunodiffusion was performed as described by Chelack et al. (1993). The single RID plates contained agarose gel with incorporated anti-bovine IgG (Bethyl Laboratories Inc., Montgomery, USA). Wells of 2 mm size were punched out of the agarose gel. Serum samples and reference serum were applied in serial dilutions with PBS-Tween buffer into the wells of the RID plates, from where it enters circularly into the gel via diffusion. The resulting immunoprecipitin ring diameters were measured with an imaging analyzer and a standard curve was generated with the reference serum. The IgG concentration of the serum samples was determined by regressing the immunoprecipitin ring diameters on the standard curve. If the IgG concentration of a sample was outside the lowest or highest standard sera the sample was retested at a lower or higher dilution that would place it within the range of the standards.

4.4.3 Data Collection and Statistical Analyses

The TP in g/dL and refractometry results in % Brix from centrifuged serum and centrifuged or filtered plasma were plotted against the IgG concentration in mg/mL obtained by RID, which was defined as the gold standard. Furthermore, the IgG concentration in mg/mL analyzed by ELISA and CE was plotted against the IgG concentration in mg/mL obtained by RID. Pearson correlation coefficients, r, were determined using these distribution plots. Correlation coefficients and Bland-Altman plots were generated using Excel (Office 2010, Microsoft Deutschland Ltd., Munich, Germany). The Bland-Altman plot was used to quantify the agreement between two quantitative measurements by using statistical limits of agreement, which were calculated by using the mean and the standard deviation (**SD**) of the differences between these two measurements (Bland and Altman, 1999; Bland and Altman, 2003). The quantitative difference of the two measurements were plotted against the average of the two measurements (RID and ELISA; RID and CE). The limits of agreement were expressed as the mean difference ± 1.96 SD (Bland and Altman, 1999; Bland and Altman, 2003). By definition, 95% of the data points lie within ± 1.96 SD of the mean difference. The limits of agreement should be discussed in light of their clinical relevance.

For all the different media, the average IgG concentration (means ± SD) and test characteristics (sensitivity, specificity, positive predictive value and negative predictive value, area under the curve) were calculated using MedCalc software (version 15.6.1, MedCalc, Mariakerke, Belgium). The definition of sensitivity (**Se**) described the probability of a test result correctly indicating FPT for a sample with IgG < 10 mg/mL. Specificity (**Sp**) described the probability of a test result correctly indicating successful passive transfer for a sample with IgG ≥ 10 mg/mL. The positive predictive value (**PPV**) was considered as a predictive probability of a test result correctly indicating FPT for a sample with IgG < 10 mg/mL. The negative predictive value (**NPV**) was considered as a predictive probability of a test result correctly indicating successful passive transfer for a sample with IgG ≥ 10 mg/mL.

By plotting the true positive rate against the false positive rate at 0.1 g/dL and 0.1% Brix increments, a receiver operating characteristic (**ROC**) curve was generated and the optimal thresholds were assessed. The optimal threshold was defined as the point on the curve with the highest combined sensitivity and specificity and its deduction was based on the area under the curve (**AUC**) according to Swets (1988) as perfect (AUC = 1), highly accurate (0.9 < AUC < 1), very accurate

(0.7 < AUC < 0.9), accurate (0.5 < AUC < 0.7), and as non-informative (AUC = 0.5). A significant statistical difference was defined for variables between the levels of a classification when $P < 0.05$; a statistical tendency was specified as differences between $P \geq 0.05$ and $P \leq 0.10$.

4.5 Results

4.5.1 Descriptive Statistics

The RID analysis identified 59 (27%) out of 216 calves with serum IgG concentrations < 10 mg/mL and 157 calves (73%) with serum IgG concentrations ≥ 10 mg/mL. The mean IgG concentration was 17.1 ± 9.8 mg/mL and the range was 0.8 to 47.8 mg IgG/mL (Table 1).

In the present study, the mean IgG concentration assessed with ELISA was 8.6 ± 4.5 mg/mL and the range was 0.3 to 21.5 mg IgG/mL. For CE the mean IgG concentration was 11.0 ± 5.7 mg/mL and the recorded range was 1.2 to 32.9 mg IgG/mL. In serum, the mean % Brix was 8.2 ± 0.78% Brix and the range was 6.6 to 11.1% Brix. For centrifuged plasma, the mean % Brix was 9.0 ± 0.81% Brix, the range was 7.2 to 11.8% Brix and for filtered plasma 9.1 ± 0.84% Brix with a range of 7.3 to 11.8% Brix, respectively. The assessment via handheld optical refractometer resulted in a mean TP concentration of

5.3 ± 0.64 g/dL in serum, 5.9 ± 0.66 g/dL in centrifuged plasma and 5.9 ± 0.68 g/dL in filtered plasma, respectively. The ranges were from 4.0 to 7.4 g/dL in serum, 4.5 to 8.0 g/dL in centrifuged plasma and 4.4 to 8.4 g/dL in filtered plasma, respectively. The descriptive statistics for all the different devices and media were listed in Table 1.

4.5.2 Correlation Coefficients and Bland-Altman plots

The Pearson correlation coefficients for serum RID and ELISA, and RID and CE are r = 0.90 and r = 0.97 respectively (Figure 1). Furthermore, CE and ELISA were highly correlated with r = 0.89. Both refractometry methods, the handheld optical and digital Brix refractometer, were highly correlated with RID using either centrifuged serum, centrifuged plasma, or filtered plasma. The range of correlation coefficients was r = 0.78 to r = 0.84 (Figure 2 and 3). The refractometer methods among themselves generated highly correlated results irrespective of the used medium. The range of correlation coefficients was r = 0.92 to r = 0.99.

The Bland-Altman plots comparing the measurement of the IgG concentration in serum of calves using RID and ELISA, as well as RID and CE, are in Figure 4. On average, the IgG concentration measured by RID was 8.6 mg/mL higher

compared to ELISA and 6.1 mg/mL higher compared to CE. The limits of agreement were -3.3 and 20.4 mg/mL for ELISA, and -2.6 and 14.9 mg/mL for CE. There was, however, a systematic bias between the difference of the two methods and the mean IgG concentration.

4.5.3 Test Characteristics

The ROC curve analyses using RID as the reference value generated the test characteristics (optimal thresholds, Se, Sp, PPV, NPV and AUC) for CE, ELISA and refractometry to identify calves with FPT using three different media (Table 2).

The analyses for ELISA showed an optimal threshold at 5.4 mg IgG/mL with Se 94.9%, Sp 97.5%, and AUC of 0.99 (Table 2; Figure 5). For CE, the optimal threshold was 6.9 mg IgG/mL with 94.9% and Sp 98.7%, as well as AUC of 0.99 (Table 2; Figure 6). Test characteristics to identify calves with FPT determined by handheld optical and digital refractometer using three different media, as well as by the laboratory methods are summarized in Table 2.

Optimal thresholds to assess FPT using plasma were higher compared with serum regardless of the method of plasma extraction. The 4 different devices had comparable AUC irrespective of the used medium.

Table 1. Descriptive statistics for total protein (g/dL), percentage points Brix (% Brix) and IgG concentration (mg IgG/mL) considering centrifuged serum, centrifuged plasma, and filtered plasma.

Test	Medium	n	Mean	SD[1]	Minimum	Maximum
PA 201 Misco Brix refractometer	Centrifuged serum	216	8.2% Brix	0.78	6.6	11.1
	Centrifuged plasma	216	9.0% Brix	0.81	7.2	11.8
	Filtered plasma	216	9.1% Brix	0.84	7.3	11.8
Handheld optical refractometer	Centrifuged serum	216	5.3 g/dL	0.64	4.0	7.4
	Centrifuged plasma	216	5.9 g/dL	0.66	4.5	8.0
	Filtered plasma	216	5.9 g/dL	0.68	4.4	8.4
Sandwich ELISA	Centrifuged serum	216	8.6 mg IgG/mL	4.52	0.3	21.5
Capillary electrophoresis	Centrifuged serum	216	11.0 mg IgG/mL	5.72	1.2	32.9
Radial immunodiffusion	Centrifuged serum	216	17.1 mg IgG/mL	9.77	0.8	47.8

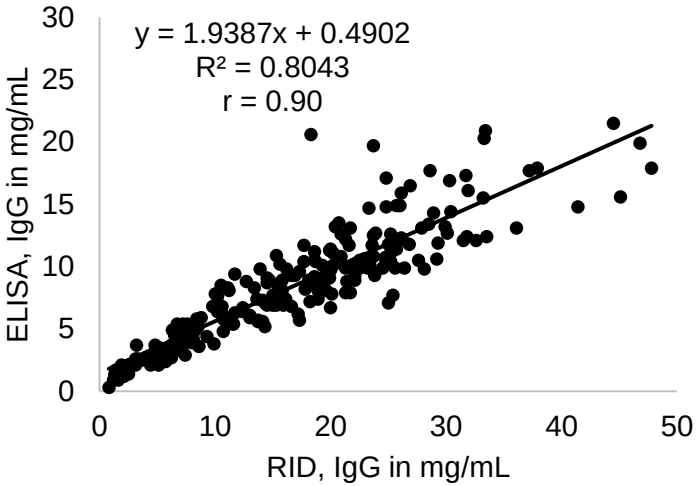
[1]SD = Standard deviation of the estimate.

Table 2. Test characteristics for IgG concentration (mg/mL) determined by ELISA and capillary electrophoresis, total protein (g/dL) and percentage points Brix (% Brix) assessed by refractometry for identification of calves with failure of passive transfer (FPT; IgG < 10 mg/mL) 1 to 7 days of age using 3 different media.

Device	Medium	n	Threshold [1]	AUC (95% CI)	*P*-value	Sensitivity	Specificity	PPV[2] (95% CI)	NPV[2] (95% CI)
PA 201 Misco Brix refractometer	Centrifuged serum	216	7.8% Brix	0.94 (0.86 – 0.97)	0.001	88.1	89.2	75.4 (63.4 – 85.0)	95.2 (90.4 – 98.1)
	Centrifuged plasma	216	8.6% Brix	0.92 (0.87 – 0.95)	0.001	86.4	82.8	65.4 (53.7 – 75.9)	94.2 (88.9 – 97.5)
	Filtered plasma	216	8.8% Brix	0.90 (0.86 – 0.94)	0.001	89.8	77.1	59.6 (48.6 – 69.9)	95.3 (90.0 – 98.2)
Handheld optical refractometer	Centrifuged serum	216	5.0 g/dL	0.92 (0.88 – 0.96)	0.001	89.8	80.3	63.1 (51.8 – 73.4)	95.5 (90.4 – 98.3)
	Centrifuged plasma	216	5.5 g/dL	0.92 (0.88 – 0.95)	0.001	83.1	84.1	66.2 (54.2 – 76.9)	93.0 (87.4 – 96.6)
	Filtered plasma	216	5.5 g/dL	0.91 (0.87 – 0.95)	0.001	81.0	86.6	69.1 (56.6 – 79.8)	92.5 (87.0 – 96.2)
Sandwich ELISA	Centrifuged serum	216	5.4 mg/mL	0.99 (0.97 – 1.00)	0.001	94.9	97.5	93.3 (83.7 – 98.2)	98.1 (94.5 – 99.6)
Capillary electrophoresis	Centrifuged serum	216	6.9 mg/mL	0.99 (0.97 – 1.00)	0.001	94.9	98.7	96.6 (88.0 – 99.6)	98.1 (94.5 – 99.6)

[1]Optimal threshold was determined by receiver operating characteristic curve analysis using the threshold with the highest sum of sensitivity and specificity to identify calves with FPT.

[2]PPV = Positive predictive value; NPV = Negative predictive value


y = 1.9387x + 0.4902
R² = 0.8043
r = 0.90
ELISA, IgG in mg/mL
RID, IgG in mg/mL
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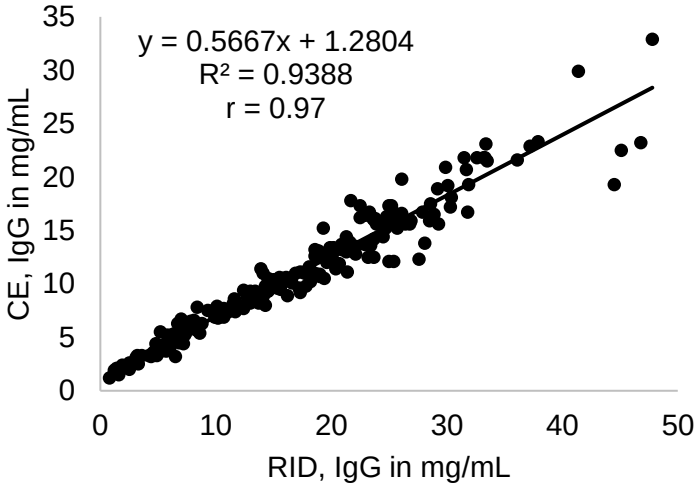
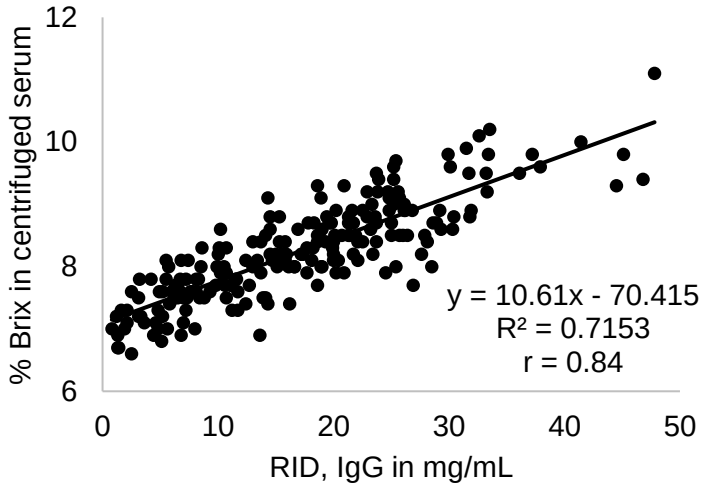
50

a)

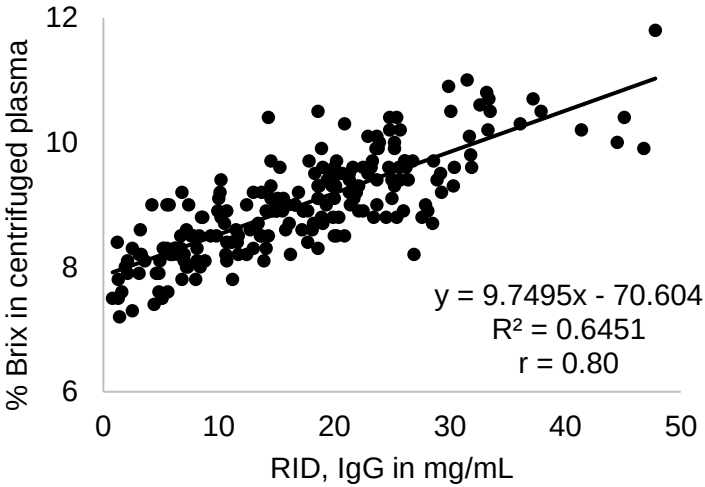

y = 0.5667x + 1.2804
R² = 0.9388
r = 0.97
CE, IgG in mg/mL
RID, IgG in mg/mL
0
5
10
15
20
25
30
35
0
10
20
30
40
50

b)

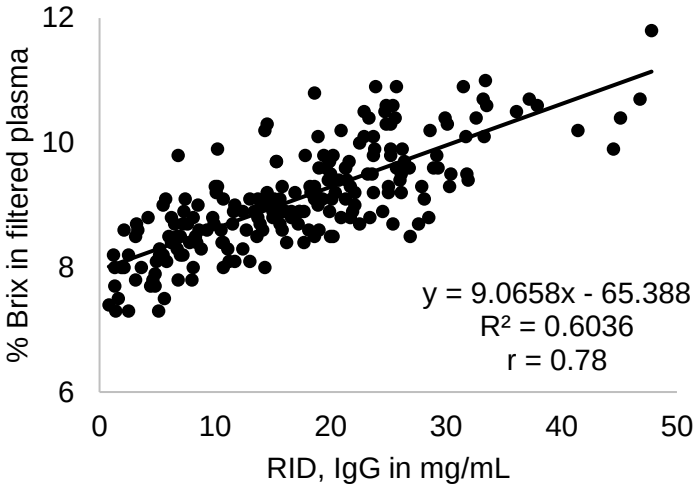
Figure 1. a) IgG concentration in centrifuged serum of calves assessed with radial immunodiffusion and compared with ELISA (n = 216; r = 0.90); **b)** IgG concentration in centrifuged serum of calves assessed with radial immunodiffusion and compared with capillary electrophoresis (n = 216; r = 0.97).


% Brix in centrifuged serum
12
10
8
6
0
10
20
30
40
50
RID, IgG in mg/mL
y = 10.61x - 70.415
R² = 0.7153
r = 0.84

a)

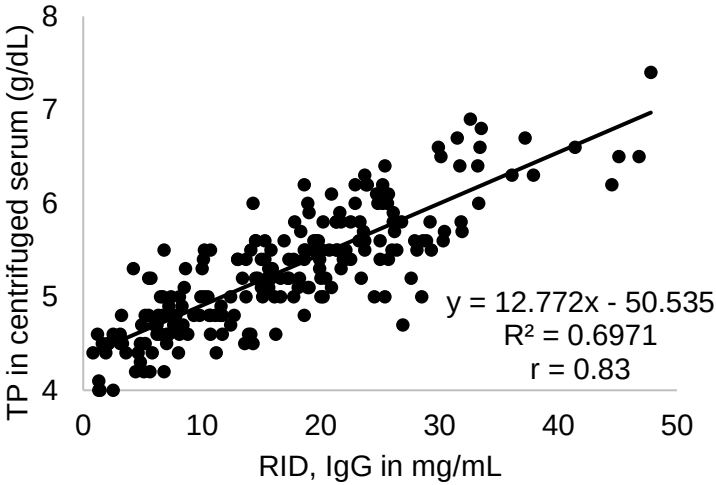

% Brix in centrifuged plasma
12
10
8
6
0
10
20
30
40
50
RID, IgG in mg/mL
y = 9.7495x - 70.604
R² = 0.6451
r = 0.80

b)

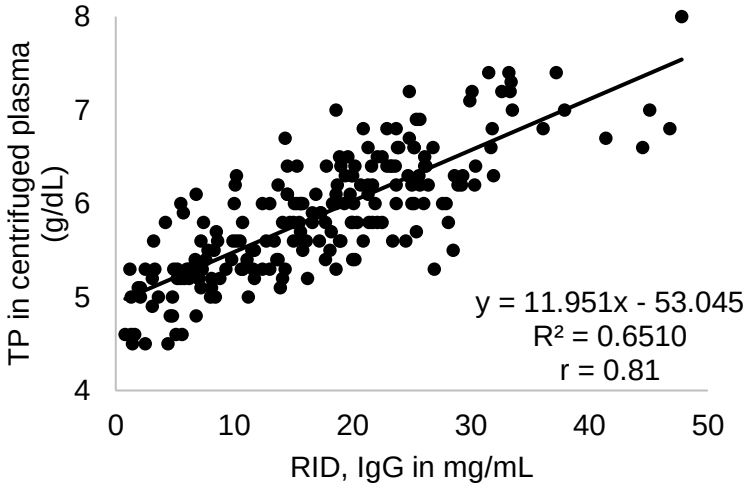


c)

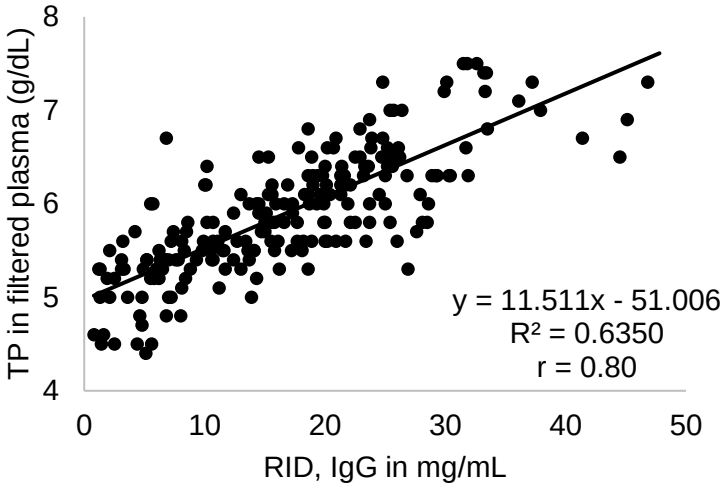
Figure 2. a) IgG concentration in centrifuged serum radial immunodiffusion (RID) compared with % Brix in centrifuged serum of calves using the Misco Brix refractometer (n = 216; r = 0.84); **b)** IgG concentration in centrifuged serum (RID) compared with % Brix in centrifuged plasma of calves using the Misco Brix refractometer (n = 216; r = 0.80); **c)** IgG concentration in centrifuged serum (RID) compared with % Brix in filtered plasma of calves using the Misco Brix refractometer (n = 216; r = 0.78).


TP in centrifuged serum (g/dL)
8
7
6
5
4
0
10
20
30
40
50
RID, IgG in mg/mL
y = 12.772x - 50.535
R² = 0.6971
r = 0.83

a)

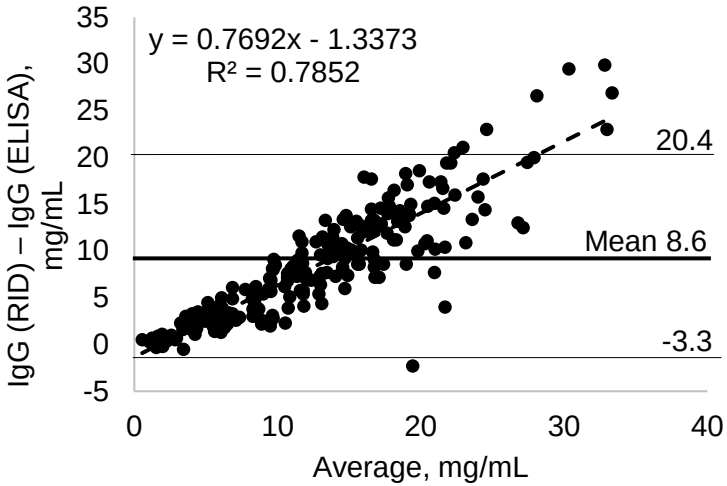

TP in centrifuged plasma (g/dL)
8
7
6
5
4
0
10
20
30
40
50
RID, IgG in mg/mL
y = 11.951x - 53.045
R² = 0.6510
r = 0.81

b)

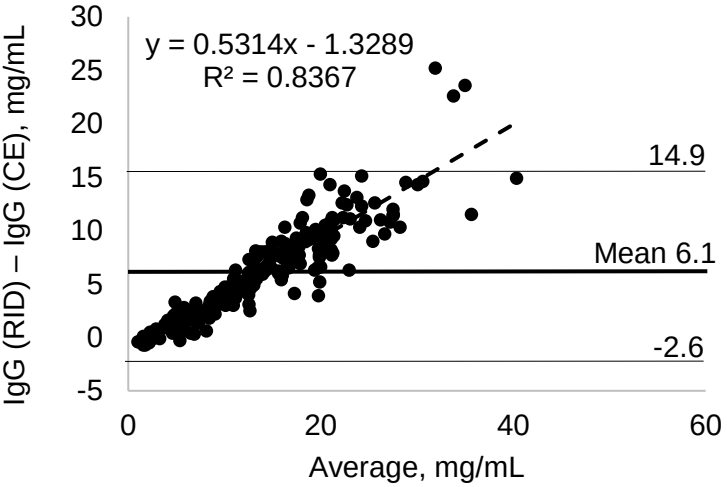


c)

Figure 3. a) IgG concentration in centrifuged serum radial immunodiffusion (RID) compared with total protein (TP) concentration in centrifuged serum of calves using the handheld optical refractometer (n = 216; r = 0.83); **b)** IgG concentration in centrifuged serum (RID) compared with TP concentration in centrifuged plasma of calves using the handheld optical refractometer (n = 216; r = 0.81); **c)** IgG concentration in centrifuged serum (RID) compared with TP concentration in filtered plasma of calves using the handheld optical refractometer (n = 216; r = 0.80).


y = 0.7692x - 1.3373
R² = 0.7852
20.4
Mean 8.6
-3.3
IgG (RID) – IgG (ELISA), mg/mL
Average, mg/mL
35
30
25
20
15
10
5
0
-5
0
10
20
30
40

a)


y = 0.5314x - 1.3289
R² = 0.8367
14.9
Mean 6.1
-2.6
IgG (RID) – IgG (CE), mg/mL
Average, mg/mL
30
25
20
15
10
5
0
-5
0
20
40
60

b)

Figure 4. a) Bland-Altman plot comparing measurement of the IgG concentration in serum of calves using 2 devices (radial immunodiffusion and ELISA). On average, the IgG concentration measured by radial immunodiffusion was 8.6 mg/mL higher compared to ELISA. The limits of agreement were -3.3 and 20.4 mg/mL (± 1.96 SD; 95% confidence limit); **b)** Bland-Altman plot comparing measurement of the IgG concentration in serum of calves using 2 devices (radial immunodiffusion and capillary electrophoresis). On average, the IgG concentration measured by radial immunodiffusion was 6.1 mg/mL higher compared to capillary electrophoresis. The limits of agreement were -2.6 and 14.9 mg/mL (± 1.96 SD; 95% confidence limit).

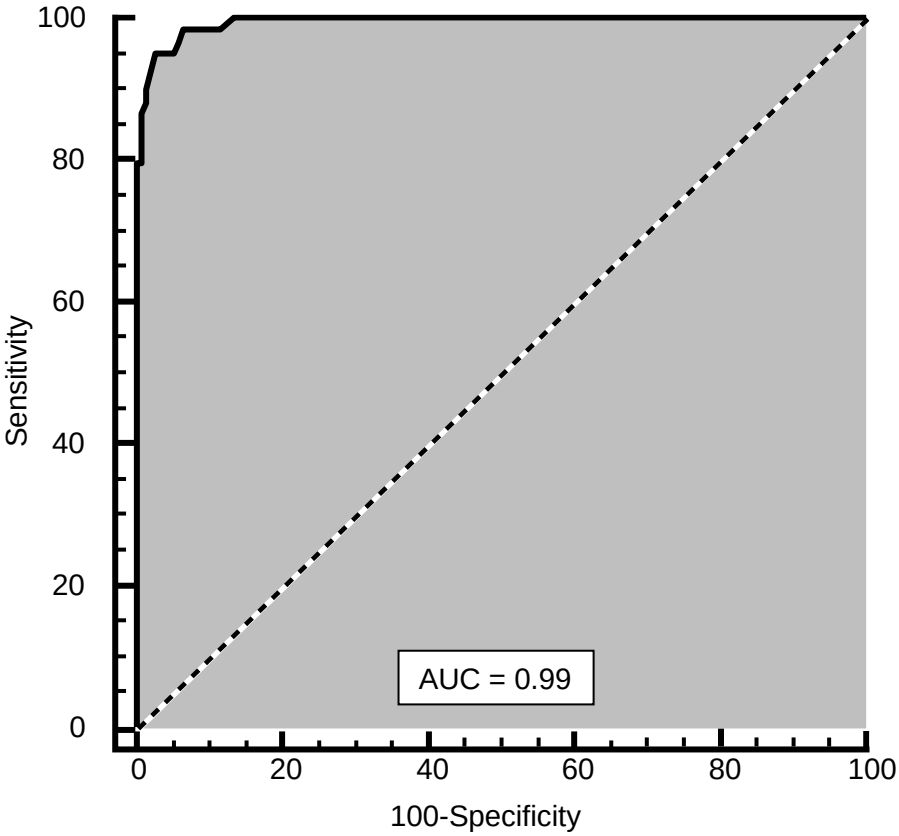


Figure 5. Receiver operating characteristic (ROC) curve for the ELISA analysis (radial immunodiffusion as reference value) measuring the IgG concentration in serum of calves. Using 5.4 mg/mL as critical threshold for the ELISA analysis, the area under the curve (AUC) result is highly accurate (AUC = 0.99) for the assessment of failure of passive transfer in dairy calves.

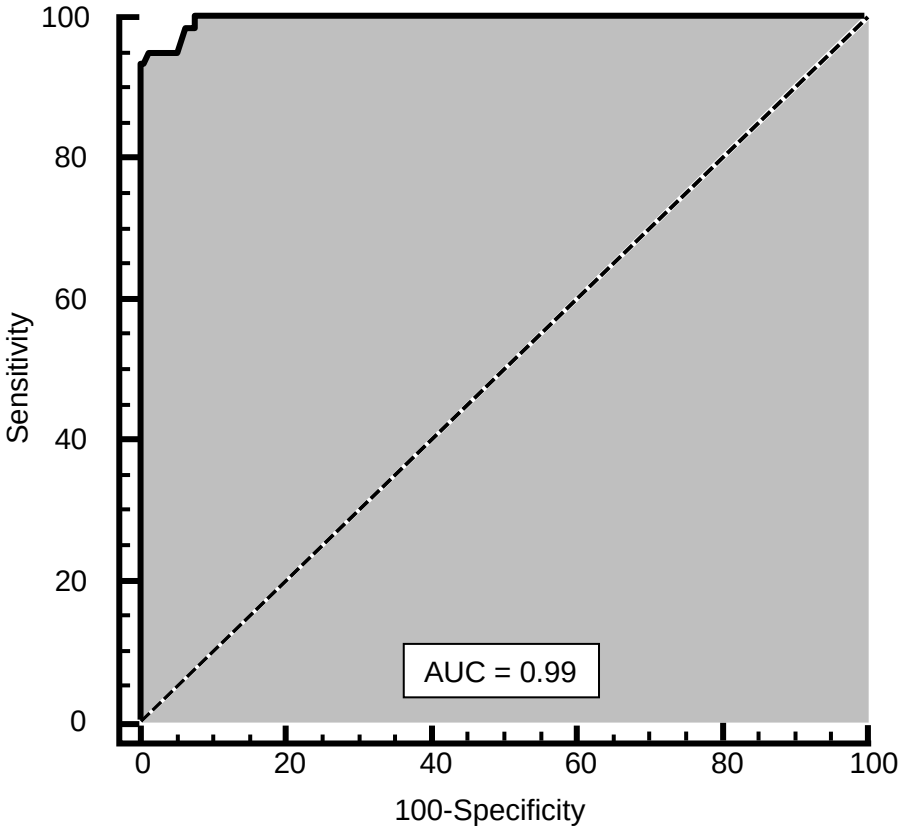


Figure 6. Receiver operating characteristic (ROC) curve for the ELISA analysis (radial immunodiffusion as reference value) measuring the IgG concentration in serum of calves. Using 6.9 mg/mL as critical threshold for the capillary electrophoresis analysis, the area under the curve (AUC) result is highly accurate (AUC = 0.99) for the assessment of failure of passive transfer in dairy calves.

4.6 Discussion

The principal findings of this study were that the four different analytical methods to assess FPT (i.e., ELISA, CE, Brix refractometer and handheld optical refractometer) were highly correlated to the gold standard RID and had a comparable AUC. Furthermore, the three different media (i.e., centrifuged serum, centrifuged plasma and filtered plasma) had highly accurate AUC results and were as well highly correlated to RID. Additionally, the optimal thresholds to assess FPT using plasma were higher compared with serum regardless of the method of plasma extraction.

4.6.1 Correlation Coefficients and Bland-Altman plots

The correlation coefficients in serum between the laboratory methods RID, ELISA and CE (mg IgG/mL) were high ($r = 0.90$ and $r = 0.97$). This is in agreement with findings of previous studies (Lee et al., 2008; Dunn et al., 2018). Our findings agree with results of previous studies, specifically a study by Bennett et al. (1994) that reported a high correlation coefficient of $r = 0.99$ by plotting the bovine IgG against molecular weight standards assessed by CE.

Gelsinger et al. (2015) reported a weaker, but still significant relationship between a modified ELISA (Bethyl

Laboratories) and a commercially available single RID assay (Triple J Farms, Bellingham, Washington, USA) using plasma IgG concentrations ($r = 0.77$; $R^2 = 0.59$; $P < 0.01$). The authors showed that the ELISA IgG concentrations in plasma samples were significantly lower compared to RID and did not recommend a direct comparison of ELISA and RID due to the risk of underestimating the IgG concentration by ELISA analysis. Furthermore, the authors proposed that a new threshold should be established for ELISA testing to determine FPT in neonatal calves, which is consistent with the findings in our study.

Serum TP, % Brix and IgG concentration determined by RID in our study were correlated ($r = 0.83$ and $r = 0.84$) which is consistent with previous studies (Calloway et al., 2002; Moore et al., 2009; Morrill et al., 2013; Deelen et al., 2014; Hernandez et al., 2016; Cuttance et al., 2017). We achieved comparable results with the additional media, centrifuged ($r = 0.81$ and $r = 0.80$) and filtered plasma ($r = 0.80$ and $r = 0.78$). In addition, there was a high correlation between the 3 different media (serum, centrifuged and filtered plasma) using either the handheld optical refractometer or the digital Brix refractometer (range of correlation coefficients: $r = 0.92$ to $r = 0.99$). This confirms that centrifuged and filtered plasma are suitable for FPT

assessment in calves as suggested by da Costa Corrêa Oliveira et al. (2019).

The Bland-Altman plots illustrated a positive difference for both alternative methods (ELISA and CE) indicating that results from ELISA and CE were lower compared with RID (8.6 mg/mL, ELISA; 6.1 mg/mL, CE). Furthermore, the limits of agreement for both methods were wide (-3.3 and 20.4 mg/mL, ELISA; -2.6 and 14.9 mg/mL, CE), due to a high mean difference and SD in both methods. The discrepancy between two measurements (RID and ELISA; RID and CE) increased with concentration. An increased variability of the differences can often be observed with increasing concentrations of the measured values (Grouven et al. 2007). The reason remains speculative. Nevertheless, a direct quantitative comparison between RID and ELISA or CE is inadequate, but both methods allow a qualitative categorization (i.e., FPT vs. successful passive transfer) when their own specific cutpoints are used and generate precise results for FPT assessment in neonatal calves.

4.6.2 Test Characteristics

The laboratory methods, ELISA and CE, were highly accurate ($0.9 < AUC < 1$) using the AUC as an indicator of overall test characteristics (Swets, 1988). In both methods, the 95% CI

did overlap, proving a precise analytical method. Furthermore, high sensitivity and specificity was detected for both methods (Se 94.9% and Sp 97.5%, ELISA; Se 94.9% and Sp 98.7%, CE). In addition, the AUC results of the two on-farm tests also demonstrated high accurancy ($0.9 < AUC < 1$) irrespective of the medium used (Swets, 1988). Our findings are comparable to the study conducted by Lee et al. (2008), which compared direct and indirect methods for FPT assessment. Lee et al. (2008) detected that the direct methods were slightly more precise than the indirect methods, as the findings of our study confirm. The two different laboratory methods (CE and ELISA) were more accurate to identify calves with FPT compared with the two different on-farm devices irrespective of the media. There was, however, no difference in test accuracy among the laboratory methods and among the on-farm devices, because the 95% CI for the AUC overlap. Furthermore, studies using a handheld optical refractometer or a digital Brix refractometer to assess FPT in calves, reported similar overall test characteristics as in our study (Elsohaby et al., 2015; Hernandez et al., 2016; Cuttance et al., 2017; Buczinski et al., 2018).

Based on our results, the new cutoff value for ELISA analysis with serum to identify calves with successful passive transfer is 5.4 mg IgG/mL, for CE analysis it is 6.9 mg IgG/mL.

This is considerably below the cutoff value of 10 mg/mL when tested with RID (Tyler et al., 1996; Weaver et al., 2000; McGuirk and Collins, 2004; Godden, 2008). Nevertheless, both laboratory methods have excellent test characteristics using these modified cutoff values.

The on-farm tests, handheld optical refractometer and Brix refractometer, revealed slightly lower cutpoints using serum (5.0 g/dL and 7.8% Brix) in comparison with other studies (5.5 g/dL and 8.3% Brix, Elsohaby et al., 2015; 5.2 g/dL and 8.5% Brix, Hernandez et al., 2016; 5.2 g/dL and 8.8% Brix, Cuttance et al., 2017). The reason for this remains speculative. These studies also used ROC curve analysis and the serum IgG concentration assessed by RID (Elsohaby et al., 2015; Hernandez et al., 2016) or turbidimetric immunoassay (**TIA**) (Cuttance et al., 2017) as reference to determine the best threshold. The optimal thresholds for centrifuged and filtered plasma were higher in comparison to serum, irrespective of the device (5.5 and 5.5 g/dL; 8.6 and 8.8% Brix), which is comparable to the findings of the study conducted by da Costa Corrêa Oliveira et al. (2019) and Elsohaby et al. (2019). Coagulation proteins, such as fibrinogen, are present in the plasma TP. These proteins are clotted during serum processing, thus leading to lower serum TP results (Lumeij and Maclean,

1996; George, 2001; da Costa Corrêa Oliveira et al., 2019; Elsohaby et al., 2019). Furthermore, plasma tubes contain anticoagulants, such as lithium heparin, which can falsely increase plasma TP concentrations if the blood tubes are underfilled (Dubin and Hunt, 1978).

4.7 Study Limitations

A limitation of our study is RID being the reference method. While several authors have consistently used RID as gold standard to validate, it is obvious that this method has some limitations. Low antigen concentration can reduce accuracy and an uneven spread of the immunoprecipin ring diameters can confound results. To achieve comparable and reproducible results, it is necessary to measure the immunoprecipitin rings exactly at the same time (Varley et al., 1985). Therefore, it is important to realize that a new and potentially better test method can be only as good as the reference method.

4.8 Conclusions

In conclusion, all four analytical methods were suitable to assess FPT in neonatal calves independent of the medium used (i.e., centrifuged serum, centrifuged plasma, filtered plasma). These different analytical methods produce results that are highly

correlated with RID (gold standard) for assessing FPT in neonatal calves, which were fed whole colostrum for first feeding. Different cutoff values must be considered, especially, if different media are used. Serum and plasma can be used interchangeably when different cutoff values are considered. This study is the first to describe that CE generates precise results for FPT assessment in neonatal calves.

4.9 Acknowledgements

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5 DISCUSSION

The overall objectives of this thesis were (1) to evaluate a filter system to harvest plasma for identification of failure of passive transfer in newborn calves, (2) to evaluate two different treatment procedures after calving to improve harvesting of high-quantity and high-quality colostrum, and (3) to evaluate different analytical methods to assess failure of passive transfer in neonatal calves.

The results of the first study indicate that a filter system to facilitate FPT assessment as a point-of-care analysis is suitable without the need of centrifugation, but for a solid recommendation, further research is warranted.

In the first study 67 neonatal calves out of 227 (30%) had FPT. Although the prevalence of FPT is not representative for the general population, because the enrolled farms were not randomly selected, a more simple way to test FPT in neonatal calves without the need of a centrifuge would be beneficial for dairy farms. It would help to implement the assessment of FPT as a calf-side monitoring tool and integrate the procedure into their daily routines. Nevertheless, the results of the first study have shown that the agreement of refractometry, including the filter system, with the chosen gold standard (ELISA) was lower than in other studies (Deelen et al., 2014; Elsohaby et al., 2015;

Hernandez et al., 2016; Cuttance et al., 2017). The Pearson correlation coefficients ranged from $r = 0.43$ to $r = 0.58$ regardless of which medium (i.e. centrifuged serum, centrifuged plasma, and filtered plasma) was used. According to Weaver et al. (2000), the ELISA measurement was similar to RID in accuracy. On this account, we used it as gold standard for the first study. However, further studies found only weak to moderate correlations between ELISA and RID for FPT analysis (Gelsinger et al., 2015; Dunn et al., 2018). The IgG concentration was significantly lower when it was assessed by ELISA compared with RID, which is consistent with the results of Gelsinger et al. (2015). We assume that this might be a reason for the weak correlations of refractometry found in our study compared with the serum IgG concentrations measured by ELISA. Therefore, the results of our first study are limited with regard to comparability to other studies using RID. For this reason, we had incorporated the filtered plasma as further medium into the study design of our third study in order to confirm the validation of the filter.

Nonetheless, the AUC results of the three different refractometry devices and media showed to be very accurate ($0.7 < AUC < 0.9$; Swets, 1988). The AUC results ranged from 0.8 to 0.85 and 95% CI did overlap, indicating a good qualitative test. Similar results were achieved in other studies using a

handheld optical refractometer (Lee et al., 2008; Elsohaby et al., 2015; Hernandez et al., 2016; Cuttance et al., 2017) or a digital Brix refractometer (Morrill et al., 2013; Elsohaby et al., 2015; Hernandez et al., 2016; Cuttance et al., 2017) for the assessment of FPT in neonatal calves.

The purpose of the second study was to evaluate two different treatment procedures at the first milking after calving to increase colostrum quantity and improve colostrum quality by applying exogenous oxytocin or by stimulating endogenous oxytocin secretion through the presence of the calf at first milking.

Although the treatment procedure did not affect colostrum quantity, the application of oxytocin and the presence of the calf increased IgG concentrations in colostrum. This might be presumably due to a high concentration of oxytocin in the bloodstream, which might have affected the integrity of the mammary tight junctions. It could have led to an increased permeability and higher IgG transfer into the udder, which is likely due to a disruption of the cell-cell contact caused by the sudden alveolar contraction triggered by oxytocin (Stelwagen and Singh, 2014). A second explanation for higher IgG concentrations in colostrum by applying exogenous oxytocin or by stimulating endogenous oxytocin secretion through the presence of the calf might be that high concentrations of oxytocin led to the removal of residual milk from the udder (Nostrand et al., 1991). This might

possibly affect colostrum quality. Although we could not detect a difference in colostrum quantity for cows receiving exogenous oxytocin or stimulation of endogenous oxytocin release.

Furthermore, the results indicated that parity was associated with increased colostral IgG concentration. Colostrum of older cows had higher IgG concentrations, probably due to the fact that older cows were being exposed to antigens for a longer time during their life than younger cows. These antibodies are transferred into the udder at the time of colostrogenesis (Conneely et al., 2013).

In addition, the data showed that cows calving during the night had the highest quantity and quality of colostrum compared with cows calving in the morning or afternoon. Similar results were found in cows calving on Sunday. We assume that these findings were associated with lower stress levels of the cows calving at night or Sunday, due to less farm activities. The IgG concentration in colostrum was reduced by a longer time interval between calving and colostrum collection, as confirmed by other studies (Moore et al., 2005; Morin et al., 2010).

Considering that the study was carried out on only one farm, the external validity is limited. Therefore, the findings need to be confirmed by a multicentric study design.

The aim of the third study was to evaluate different analytical methods to assess FPT in neonatal calves. The

prevalence of FPT in this study was 27% (59 of 216 calves had FPT). Again, the prevalence of FPT does not correspond to the general population, since only two farms were enrolled and these were not randomly selected.

All four different analytical methods (i.e. ELISA, CE, two refractometry methods) were highly correlated in comparison to the gold standard RID and generated highly accurate AUC results ($0.9 < AUC < 1$; Swets, 1988). The range of Pearson correlation coefficients was $r = 0.78$ to $r = 0.97$ irrespective of the device and medium. The laboratory methods (RID vs. ELISA and RID vs. CE) had very high correlation coefficients ($r = 0.90$ and $r = 0.97$), which is consistent with previous studies (Lee et al., 2008; Dunn et al., 2018). Bennett et al. (1994) plotted bovine IgG against molecular weight standards using CE and found a high correlation coefficient ($r = 0.99$).

Gelsinger et al. (2015) reported a weaker correlation coefficient between ELISA and RID ($r = 0.77$; $R^2 = 0.59$; $P < 0.01$) than we found in our study. The authors rejected the direct comparison of ELISA and RID due to the risk of underestimating the IgG concentration by ELISA testing. In the Bland-Altman plots, we were also able to demonstrate a positive difference for ELISA and CE measurements, indicating that results from ELISA and CE were lower compared with RID. This is in agreement that a direct quantitative comparison between

RID and ELISA or CE is inappropriate, but both methods generate precise results for a qualitative categorization (i.e., FPT vs. successful passive transfer) and are therefore suitable for the assessment of FPT.

Gelsinger et al. (2015) recommended that new thresholds should be established for ELISA testing to determine FPT in neonatal calves, which is in accordance with the results of our study.

Therefore, all analytical methods were appropriate to assess FPT in neonatal calves, regardless of the used medium (i.e., centrifuged serum, centrifuged plasma, filtered plasma). We recorded slightly more precise results with the direct laboratory methods (i.e., ELISA and CE) than with the indirect methods. This is in agreement with others (Lee et al., 2008). Nevertheless, the test accuracy within the direct laboratory methods was very good, as the 95% CI for AUC overlapped. The indirect on-farm devices had also a very good test accuracy, as the 95% CI for AUC within these tests overlapped as well. Anyhow, different cutoff values must be considered for the FPT assessment with different analysis methods and different media. This is the first study to report that CE provides reliable diagnoses for FPT analysis in neonatal calves.

Ultimately, despite of advances in newborn calf care and colostrum management, the high prevalence of FPT [27 – 30%

in our studies and 38.8% assessed by McMorran (2006)] is still unsustainably high on dairy farms in Germany and the US (NAHMS, 1993; NAHMS, 2007). Preventive strategies should be enforced more widely on dairy farms. Herd management strategies such as standardized procedures for hygienic milking and storage of the first colostrum, routine control of colostrum quality, standardized procedures for the delivery of the first meal, and regular control of success through FPT monitoring should be implemented to improve perinatal welfare. These findings presented herein help to raise awareness of this neglected problem of FPT in newborn dairy calves, supporting the development of on-farm solutions that can be implemented into the daily work routine of dairy farms.

Radial immunodiffusion is considered the best gold standard for FPT measurement, although this method has some limitations. Low antigen concentration can reduce its accuracy, and an uneven spread of the immunoprecipin ring diameters can confound results. It is necessary to measure the immunoprecipitin rings exactly at the same time to achieve reproducible results (Varley et al., 1985). Implementing another gold standard requires a diagnostic randomized controlled trial, which is rarely conducted for the assessment of diagnostic tests. Such an approach implies a randomized comparison of two diagnostic tests (reference test vs. experimental test). The

therapeutic interventions must be identical and are based on the results of the competing diagnostic tests, i.e. illness: yes or no (Rogder et al., 2012). Such a diagnostic randomized trial, however, is challenging (i.e., study design, sample size) to conduct for the assessment of FPT. This requires explicit standardized procedures for colostrum feeding and disease monitoring in order to make the comparison possible.

6 SUMMARY

Diagnosis of failure of passive transfer in dairy calves and procedures after calving to improve harvesting of high-quantity and high-quality colostrum

The overall objectives of this thesis were (1) to evaluate a filter system to harvest plasma for identification of failure of passive transfer in newborn calves, (2) to evaluate 2 different treatment procedures after calving to improve harvesting of high-quantity and high-quality colostrum, and (3) to evaluate different analytical methods to assess failure of passive transfer in neonatal calves.

To validate a filter system to harvest plasma for the assessment of failure of passive transfer (FPT) in neonatal calves, venous blood samples (serum and plasma) were withdrawn from 227 Holstein Friesian calves aged 1 to 7 d. Serum IgG concentrations were determined using a sandwich enzyme-linked immunosorbent assay (ELISA) (gold standard), and failure of passive transfer was defined as IgG concentrations below 10 mg/mL. Serum was obtained through centrifugation, and plasma was extracted either through centrifugation or a disposable filter system (2-Drop-Filter, Pharmadoc, Lübeck, Germany). To assess the total proteins (TP) and total solids (TS)

in serum and plasma, a handheld optical refractometer (Euromex Holland, Arnhem, the Netherlands) and 2 digital Brix refractometers (device 1: HI 96801 digital refractometer, Hanna Instruments, Woonsocket, RI; device 2: Misco PA201, Misco, Solon, OH) were used. The optimal threshold for each device and each medium, i.e centrifuged serum, centrifuged plasma, and filtered plasma, was calculated using the receiver operating characteristic (ROC) curve analyses.

The prevalence of failure of passive transfer in neonatal calves was 30% (67/227). The optimal threshold for the handheld optical refractometer using serum was 5.6 g/dL [sensitivity (Se) 70.1%; specificity (Sp) 80.0%; area under the curve (AUC) 0.85]. For centrifuged plasma, the optimal threshold was higher at 6.3 g/dL (Se 82.1%; Sp 68.1%; AUC 0.84), and for filtered plasma, the threshold was 6.0 g/dL (Se 56.7%; Sp 90.0%; AUC 0.80). The digital Brix refractometer (device 1: HI 96801) had the optimal threshold at 8.9% Brix using serum (Se 82.1%; Sp 63.8%; AUC 0.81), and at 9.4% Brix (Se 76.1%; Sp 73.7%; AUC 0.80) using centrifuged plasma, respectively. The device 2 (Misco PA201) determined the optimal threshold at 8.7% Brix (Se 74.6%; Sp 76.2%; AUC 0.83), 9.5% Brix (Se 80.6%; Sp 70.6%; AUC 0.83), and 9.2% Brix (Se 58.2%; Sp 87.5%; AUC 0.80) using serum, centrifuged plasma, and filtered plasma, respectively. All the three devices with the different media had

comparable test characteristics to assess calves with FPT, based on the AUC determined with ROC curve analyses.

Plasma samples (centrifuged and filtered plasma) revealed higher optimal thresholds to assess FPT compared with serum. Furthermore, the three different devices had comparable AUC irrespective of the used medium (centrifuged serum, centrifuged plasma, or filtered plasma). The results demonstrate that a filter system is suitable as a point-of-care analysis for FPT assessment, but further research is necessary.

The aim of the second study was to evaluate two different treatment procedures at first milking after calving to increase colostrum quantity and to improve colostrum quality in dairy cows. We assumed that exogenous treatment with oxytocin or the presence of the calf at harvesting the first colostrum would lead to higher colostrum quantity and higher IgG concentrations. For the study, a total of 567 dairy cows were enrolled. For the final analyses, only 521 animals were considered and 46 dairy cows were excluded due to several reasons, such as lameness, bloody or mastitic colostrum, gestation length of less than 265 d, twin births and missing data. Three groups were built in which the cows were randomly assigned on a daily basis: (1) control group (CON; n = 177), (2) application of 20 IU of oxytocin i.m. (OXY; n = 163), and (3) presence of the calf (CA; n = 181) before and during milking. The milking of the first colostrum took place

in a separate milking parlor. Dairy cows in the control and oxytocin group had no contact with their calves after calving. Three minutes before manual stimulation, the cows in the oxytocin group were injected with 20 IU of oxytocin i.m. into the neck region. Cows in the CA group got their calf presented three minutes before milking, whereby the calf was placed into a calf cart and located in front of the cow.

Colostrum quantity (kg) was recorded and colostrum quality was determined by Brix refractometry and ELISA. To evaluate the data a generalized linear mixed model (GENLINMIXED) was constructed using SPSS (SPSS Inc., IBM, Ehningen, Germany).

The mean colostrum quantity was 4.17 ± 0.30 kg (means ± SE). The treatment procedures and the harvesting time after calving did not affect colostrum quantity, but parity, calf birth weight, and calving time had an influence on colostrum quantity. The lowest quantity of colostrum had cows in second parity (3.74 ± 0.37 kg). Whereas cows in parity 1 (4.75 ± 0.34 kg) and cows in parity 3 or greater (4.75 ± 0.38 kg) had higher colostrum quantities. Further, cows calving at night (22:00 until 06:00 h; 4.93 ± 0.37 kg) had the highest quantity of colostrum. Cows calving in the morning (06:00 until 14:00 h; 4.17 ± 0.38 kg) or afternoon (14:00 until 22:00 h; 4.14 ± 0.34 kg) had a reduced amount of colostrum.

Forty-eight percent of the colostrum samples assessed by ELISA contained ≥ 50 mg IgG/mL (54.6 ± 2.80 mg IgG/mL). The treatment procedures had an effect on colostrum quality, as well as colostrum quantity, parity, calving time, harvesting time after calving, and the calving day during the week. The treatment procedures achieved on mean higher IgG concentrations in colostrum (OXY: 57.0 mg IgG/mL; CA: 56.0 mg IgG/mL) compared with the control group (50.7 mg IgG/mL). High colostrum quantity and a longer time lag between calving and milking decreased the colostrum quality. Cows in parity 3 or greater (64.6 ± 2.59 mg IgG/mL) had higher IgG concentrations compared with cows in parity 1 (48.5 ± 2.86 mg IgG/mL) and cows in parity 2 (50.7 ± 2.89 mg IgG/ mL). Calving at night resulted in greater IgG concentrations (60.4 ± 2.92 mg IgG/mL) compared to calving at the morning or afternoon time (51.9 ± 2.98 and 51.3 ± 2.71 mg IgG/mL). Calving on Sundays increased the colostrum quality (61.4 ± 3.70 mg IgG/mL).

The treatment procedures and the harvesting time after calving had no effect on colostrum quality assessed by Brix refractometry. Nevertheless, a negative association was observed between colostrum quantity and quality as with ELISA testing. Cows in parity 3 or greater showed higher Brix readings (27.7 ± 0.26% Brix) compared with cows in parity 1 (25.3 ± 0.30% Brix) and cows in parity 2 (25.0 ± 0.32% Brix).

The purpose of the third study was to evaluate different analytical methods [i.e., ELISA, capillary electrophoresis (CE), refractometry] and three different media (i.e., centrifuged serum, centrifuged plasma, filtered plasma) to assess FPT in neonatal calves. As gold standard, radial immunodiffusion (RID) was chosen, and as before, FPT was defined by serum IgG concentrations < 10 mg/mL. Blood samples were collected from Holstein Friesian calves (n = 216) aged 1 to 7 days, from two commercial dairy herds in Northeast Germany. Serum was gained through centrifugation, and plasma extraction was performed either through a filter system or through centrifugation, as in the first study. For plasma filtration a disposable plasma filter was used (2-Drop-Filter, Pharmadoc, Lübeck, Germany). The laboratory methods, RID, ELISA, and CE, determined the IgG concentration in serum samples. For refractometry, two refractometers were used, a handheld optical refractometer (RF.5612 Handheld refractometer, Euromex Holland, Arnhem, Netherlands), assessing the TP concentration in all three media, and a digital Brix refractometer (Misco PA201, Misco, Solon, OH), assessing the TS, respectively.

The RID analysis showed a prevalence of FPT of 27% (59/216) and 73% (157/216) with successful passive transfer. The Pearson correlation coefficient between RID and CE in serum was r = 0.97, and between RID and ELISA, it was r = 0.90,

respectively. In addition, a high correlation between CE and ELISA could be identified (r = 0.89). Refractometry results were highly correlated with RID using either centrifuged serum, centrifuged plasma, or filtered plasma (Brix refractometer: r = 0.84; r = 0.80; r = 0.78; handheld optical refractometer: r = 0.83; r = 0.81; r = 0.80). The test characteristics to identify calves with FPT (optimal thresholds, Se, Sp, positive predictive value [PPV], negative predictive value [NPV], and AUC) for all methods and the three different media were determined by ROC curve analyses by using RID as the reference value.

In summary, all four different analytical methods were suitable to assess FPT (ELISA, CE, and two refractometry methods). The test accuracy within the direct laboratory methods and within the indirect on-farm devices was very good, as the 95% CI for AUC overlapped, regardless of the three different media (centrifuged serum and plasma, or filtered plasma). However, different cutoff values for each analytical method must be considered, in particular if different media were used. The results demonstrate again, as in the first study, that optimal thresholds using plasma were higher compared with serum. In conclusion, all methods can be used for the assessment of FPT, and serum and plasma samples can be used interchangeably if the different cutoff values are taken into consideration.

Overall, this thesis shows that (1) a disposable plasma filter system is appropriate as a point-of-care system for FPT analysis, but further research is required, (2) exogenous oxytocin and the presence of the calf improve the IgG concentration in colostrum at the first milking, but do not have an influence on colostrum quantity, and (3) different analytical methods are suitable to assess FPT if different cutoff values are taken into account. These results have the potential to improve colostrum management and calf health on dairy farms.

7 ZUSAMMENFASSUNG

Diagnostik der fehlerhaften passiven Immunisierung bei Milchkälbern mit mangelhafter Kolostrum-Versorgung und Verfahren zur Verbesserung der Kolostrum-Qualität und -Menge nach der Kalbung.

Die Ziele dieser Arbeit waren (1) ein Filtersystem zur Gewinnung von Plasma zu evaluieren, um eine fehlerhafte passive Immunisierung von neugeborenen Kälbern bedingt durch mangelhafte Kolostrum-Versorgung zu beurteilen. Des Weiteren (2) zwei verschiedene Behandlungsverfahren nach dem Abkalben zu untersuchen, um die Kolostrum-Menge und Kolostrum-Qualität zu verbessern. Sowie (3) verschiedene Analysemethoden zur Beurteilung der fehlerhaften passiven Immunisierung durch mangelhafte Kolostrum-Versorgung von neugeborenen Kälbern zu bewerten.

Um ein Filtersystem zur Plasmagewinnung für die Beurteilung der fehlerhaften passiven Immunisierung bzw. Transfers (FPT) bei neugeborenen Kälbern zu validieren, wurden venöse Blutproben untersucht. Serum- und Plasmaproben wurden von 227 Kälbern der Rasse Holstein Friesian im Alter von ein bis sieben Lebenstagen entnommen. Die IgG-Konzentration im Serum wurde als Goldstandard mittels

Sandwich Enzyme-linked Immunosorbent Assay (ELISA) bestimmt. Alle Kälber mit IgG-Konzentrationen < 10 mg/mL im Serum waren von FPT betroffen. Serum wurde durch Zentrifugation gewonnen und Plasma wurde entweder durch Zentrifugation oder durch ein Einweg-Filtersystem (2-Drop-Filter, Pharmadoc, Lübeck, Deutschland) extrahiert. Um das Totalprotein (TP) und die Gesamtfeststoffe (TS) im Serum und Plasma zu bestimmen, wurden ein tragbares optisches Refraktometer (Euromex Holland, Arnhem, Niederlande) und zwei digitale Brix-Refraktometer (Gerät 1: HI 96801 digitales Refraktometer, Hanna Instruments, Woonsocket, RI; Gerät 2: Misco PA201, Misco, Solon, OH) verwendet. Der optimale Grenzwert für jedes Gerät und jedes Medium, d.h. für zentrifugiertes Serum, zentrifugiertes und gefiltertes Plasma, wurde anhand der ROC-Kurven-Analyse (Receiver Operating Characteristics) berechnet.

Die Prävalenz von FPT bei neugeborenen Kälbern befand sich bei 30 % (67/227). Der optimale Grenzwert für das optische Refraktometer betrug 5,6 g/dL im Serum (Sensitivität [Se] 70,1 %; Spezifität [Sp] 80,0 %; Fläche unter der Kurve [AUC] 0,85). Für zentrifugiertes Plasma lag der optimale Grenzwert höher, bei 6,3 g/dL (Se 82,1 %; Sp 68,1 %; AUC 0,84) und für gefiltertes Plasma lag er bei 6,0 g/dL (Se 56,7 %; Sp 90,0 %; AUC 0,80). Das digitale Brix-Refraktometer (Gerät 1: HI 96801)

zeigte den optimalen Grenzwert von 8,9 % Brix im Serum (Se 82,1 %; Sp 63,8 %; AUC 0,81) und 9,4 % Brix (Se 76,1 %; Sp 73,7 %; AUC 0,80) im zentrifugierten Plasma an. Das zweite Gerät (Misco PA201) hatte den optimalen Grenzwert von 8,7 % Brix im Serum (Se 74,6 %; Sp 76,2 %; AUC 0,83), 9,5 % Brix im zentrifugierten Plasma (Se 80,6 %; Sp 70,6 %; AUC 0,83) und 9,2 % Brix im gefilterten Plasma (Se 58,2 %; Sp 87,5 %; AUC 0,80). Alle drei Geräte mit den verschiedenen Medien hatten vergleichbare Testeigenschaften, um Kälber mit FPT zu beurteilen, basierend auf der AUC, die mit der ROC-Kurven-Analyse bestimmt wurde.

Plasmaproben (zentrifugiertes und gefiltertes Plasma) hatten höhere optimale Grenzwerte zur Beurteilung von FPT im Vergleich zu Serumproben. Darüber hinaus zeigten die drei verschiedenen Geräte unabhängig vom verwendeten Medium (zentrifugiertes Serum, zentrifugiertes oder gefiltertes Plasma) vergleichbare AUC. Aus den Ergebnissen lässt sich schließen, dass ein Filtersystem als Point-of-Care-Analyse für die FPT-Bewertung geeignet ist, aber weitere Forschung dazu notwendig ist.

Das Ziel der zweiten Studie war es, zwei verschiedene Behandlungsverfahren beim ersten Melken nach dem Kalben zu bewerten, um die Kolostrum-Menge und die Kolostrum-Qualität bei Milchkühen zu verbessern. Unsere Hypothese lautete, dass

eine Behandlung mit Oxytocin oder das Vorhandensein des Kalbes beim ersten Melken nach der Kalbung zu einer höheren Kolostrum-Menge und zu einer höheren IgG-Konzentration führt. In die Studie gingen insgesamt 567 Milchkühe ein. Für die endgültige Auswertung wurden aber nur 521 Tiere berücksichtigt, da 46 Milchkühe aus verschiedenen Gründen ausgeschlossen wurden, z.B. Lahmheit, blutiges oder entzündlich verändertes Kolostrum, Trächtigkeitsdauer unter 265 Tagen, Zwillingsgeburten und fehlende Daten. Es wurden drei Gruppen gebildet, denen die Kühe täglich zufällig zugeordnet wurden: (1) Kontrollgruppe (CON; n = 177), (2) Anwendung von 20 IE Oxytocin i.m. (OXY; n = 163) und (3) Vorhandensein des Kalbes (CA; n = 181) vor und während des Melkens. Das Melken des ersten Kolostrums erfolgte in einem separaten Melkstand. Milchkühe in der Kontroll- und Oxytocingruppe hatten nach dem Kalben keinen Kontakt zu ihren Kälbern. Drei Minuten vor der manuellen Stimulation wurde den Kühen der Oxytocingruppe 20 IE Oxytocin i.m. in die Halsregion injiziert. Den Kühen der CA-Gruppe wurde drei Minuten vor dem Melken ihr Kalb präsentiert, wobei das Kalb in eine Transportkarre für Kälber gelegt und vor der Kuh platziert wurde.

Die Kolostrum-Menge wurde in Kilogramm erfasst und die Kolostrum-Qualität wurde mittels Brix-Refraktometrie und ELISA bestimmt. Zur Auswertung der Daten wurde mit SPSS

(SPSS Inc., IBM, Ehningen, Deutschland) ein generalisiertes lineares gemischtes Modell (GENLINMIXED) erstellt.

Die mittlere Kolostrum-Menge betrug 4,17 ± 0,30 kg (Mittelwert ± Standardfehler). Die Behandlungsverfahren und die Zeit des ersten Melkens nach dem Kalben hatten keinen Einfluss auf die Kolostrum-Menge, aber die Laktationsanzahl, das Geburtsgewicht des Kalbes und die Kalbezeit hatten einen Einfluss auf die Kolostrum-Menge. Die geringste Menge hatten Kühe in zweiter Laktation (3,74 ± 0,37 kg). Während Erstkalbinnen (4,75 ± 0,34 kg) und Kühe in Laktation 3 oder höher (4,75 ± 0,38 kg) höhere Kolostrum-Mengen aufwiesen. Weiterhin hatten Kühe, die nachts kalbten (22:00 bis 06:00 Uhr; 4,93 ± 0,37 kg), die höchste Kolostrum-Menge. Kühe, die morgens (06:00 bis 14:00 Uhr; 4,17 ± 0,38 kg) oder nachmittags (14:00 bis 22:00 Uhr; 4,14 ± 0,34 kg) kalbten, hatten eine geringere Kolostrum-Menge.

Achtundvierzig Prozent der mit ELISA untersuchten Kolostrumproben enthielten ≥ 50 mg IgG/mL (54,6 ± 2,80 mg IgG/mL). Die Qualität des Kolostrums wurde durch die Behandlungsverfahren sowie durch die Kolostrum-Menge, die Laktationsanzahl, die Kalbezeit, die Zeit des ersten Melkens nach dem Kalben und den Wochentag, an dem die Abkalbung stattfand, beeinflusst. Die Behandlungsverfahren führten zu durchschnittlich höheren IgG-Konzentrationen im Kolostrum

(OXY: 57,0 mg IgG/mL; CA: 56,0 mg IgG/mL) im Vergleich zur Kontrollgruppe (50,7 mg IgG/mL). Eine hohe Kolostrum-Menge und eine längere Zeitverzögerung zwischen Kalben und Melken verminderten die Kolostrum-Qualität. Kühe in Laktation 3 oder höher (64,6 ± 2,59 mg IgG/mL) hatten eine höhere IgG-Konzentration als Erstkalbinnen (48,5 ± 2,86 mg IgG/mL) und Kühe in Laktation 2 (50,7 ± 2,89 mg IgG/mL). Das Kalben in der Nacht führte zu höheren IgG-Konzentrationen (60,4 ± 2,92 mg IgG/mL) im Vergleich zum Kalben am Morgen oder Nachmittag (51,9 ± 2,98 und 51,3 ± 2,71 mg IgG/mL). Das Kalben an Sonntagen erhöhte die Kolostrum-Qualität (61,4 ± 3,70 mg IgG/mL).

Die Behandlungsverfahren und die Zeit des ersten Melkens nach dem Kalben hatten keinen Einfluss auf die Kolostrum-Qualität, die durch Brix-Refraktometrie beurteilt wurde. Dennoch wurde wie bei der ELISA-Untersuchung ein negativer Zusammenhang zwischen Kolostrum-Menge und -Qualität festgestellt. Kühe in Laktation 3 oder höher zeigten höhere Brixwerte (27,7 ± 0,26 % Brix) im Vergleich zu Erstkalbinnen (25,3 ± 0,30 % Brix) und Kühen in Laktation 2 (25,0 ± 0,32 % Brix).

Das Ziel der dritten Studie war es, verschiedene Analysemethoden (d.h. ELISA, Kapillarelektrophorese [CE], Refraktometrie) und drei verschiedene Medien (d.h.

zentrifugiertes Serum, zentrifugiertes Plasma, gefiltertes Plasma) zur Beurteilung von FPT bei neugeborenen Kälbern zu evaluieren. Als Goldstandard wurde die radiale Immundiffusion (RID) gewählt und FPT wurde, wie schon erwähnt, mit IgG-Konzentrationen < 10 mg/mL im Serum definiert. Blutproben wurden von Kälbern der Rasse Holstein Friesian (n = 216) im Alter von ein bis sieben Lebenstagen aus zwei kommerziellen Milchviehbetrieben in Nordostdeutschland entnommen. Das Serum wurde durch Zentrifugation gewonnen und die Plasmaextraktion wurde, wie bereits in der ersten Studie, entweder durch ein Filtersystem oder durch Zentrifugation durchgeführt. Für die Plasmafiltration wurde ein Einweg-Plasmafilter verwendet (2-Drop-Filter, Pharmadoc, Lübeck, Deutschland). Die Labormethoden, RID, ELISA und CE, bestimmten die IgG-Konzentration in den Serumproben. Für die Refraktometrie wurden zwei Refraktometer verwendet, ein optisches Refraktometer (RF.5612 Handrefraktometer, Euromex Holland, Arnheim, Niederlande) zur Beurteilung der TP-Konzentration in allen drei Medien und ein digitales Brix-Refraktometer (Misco PA201, Misco, Solon, OH) zur Beurteilung der TS.

Die RID-Analyse ergab eine FPT-Prävalenz von 27 % (59/216). Der Pearson-Korrelationskoeffizient zwischen RID und CE lag bei r = 0,97 im Serum; zwischen RID und ELISA lag er

bei r = 0,90. Darüber hinaus konnte eine hohe Korrelation zwischen CE und ELISA festgestellt werden (r = 0,89). Die Ergebnisse der Refraktometrie korrelierten hochgradig mit den RID-Ergebnissen im zentrifugierten Serum, zentrifugierten Plasma und gefilterten Plasma (Brix-Refraktometer: r = 0,84; r = 0,80; r = 0,78; optisches Refraktometer: r = 0,83; r = 0,81; r = 0,80). Die Testeigenschaften (optimale Grenzwerte, Se, Sp, positiver prädiktiver Wert [PPV], negativer prädiktiver Wert [NPV] und AUC) zur Beurteilung von Kälbern mit FPT für alle Verfahren und alle drei verschiedenen Medien wurden durch ROC-Kurven Analyse unter Verwendung des RID als Referenzwert bestimmt.

Zusammenfassend sind alle vier verschiedenen Analysemethoden zur Beurteilung von FPT (ELISA, CE und zwei Methoden der Refraktometrie) geeignet. Die Testgenauigkeit innerhalb der direkten Labormethoden und innerhalb der indirekten On-Farm-Geräte war sehr gut, da sich die 95 % CI für AUC überlappten, unabhängig von den drei verschiedenen Medien (zentrifugiertes Serum und Plasma sowie gefiltertes Plasma). Allerdings müssen für jede Analysemethode unterschiedliche Grenzwerte berücksichtigt werden, insbesondere, wenn unterschiedliche Medien verwendet werden. Die Ergebnisse zeigten erneut, wie in der ersten Studie, dass die optimalen Grenzwerte für Plasma im Vergleich zu Serum höher lagen. Folglich können alle Methoden zur

Beurteilung von FPT genutzt sowie Serum- und Plasmaproben eingesetzt werden, wenn man die unterschiedlichen Grenzwerte berücksichtigt.

Insgesamt zeigt diese Arbeit, dass (1) ein Einweg-Plasma-Filtersystem als Point-of-Care-System für die FPT-Analyse geeignet ist, aber weitere Forschungsarbeit erforderlich ist, (2) exogenes Oxytocin und das Vorhandensein des Kalbes die IgG-Konzentration im Kolostrum beim ersten Melken verbessern, aber keinen Einfluss auf die Kolostrum-Menge haben, und (3) verschiedene Analysemethoden zur Beurteilung von FPT geeignet sind, wenn unterschiedliche Grenzwerte berücksichtigt werden. Diese Ergebnisse haben das Potenzial, das Kolostrum-Management und die Gesundheit der Kälber in Milchviehbetrieben zu verbessern.

8 REFERENCES FOR INTRODUCTION AND DISCUSSION

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9 PUBLICATIONS

Research articles

da Costa Corrêa Oliveira, L., S. Borchardt, W. Heuwieser, E. Rauch, M. Erhard, and F. Sutter. (2019):
Evaluation of a filter system to harvest plasma for identification of failure of passive transfer in newborn calves.
Journal of Dairy Science. 102:557–566.
https://doi.org/10.3168/jds.2018-15006

Sutter, F., S. Borchardt, G. M. Schuenemann, E. Rauch, M. Erhard, and W. Heuwieser. (2019):
Evaluation of 2 different treatment procedures after calving to improve harvesting of high-quantity and high-quality colostrum.
Journal of Dairy Science. 102:9370-9381.
https://doi.org/10.3168/jds.2019-16524

Sutter, F., E. Rauch, M. Erhard, R. Sargent, C. Weber, W. Heuwieser, and S. Borchardt. (2019):
Evaluation of different analytical methods to assess failure of passive transfer in neonatal calves.
Journal of Dairy Science. Submitted, pending minor revision.

Oral presentations at conferences

da Costa Corrêa Oliveira, L., S. Borchardt, W. Heuwieser, E. Rauch, M. Erhard, and F. Sutter. (2019):
Evaluierung eines Plasma-Filtersystems zur Bewertung der Kolostrum-Versorgung bei neugeborenen Kälbern.
43. Leipziger Laborfortbildung – Labordiagnostik in der Bestandsbetreuung.
Leipzig. 22.06.2018.

Sutter, F., S. Borchardt, G. M. Schuenemann, E. Rauch, M. Erhard, and W. Heuwieser. (2019):
Evaluation of 2 different treatment procedures after calving to improve harvesting of high-quantity and high-quality colostrum.
ICPD-Congress,17th International Conference on Production Diseases in Farm Animals 2019
Bern. 27.06. – 29.06.2019.

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11 DECLARATION OF INDEPENDENCE

Hiermit erkläre ich, dass ich, Franziska Sutter, alle Studien selbstständig durchgeführt und die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Tabelle 1. Eigener Anteil[1] an den Forschungsprojekten der vorliegenden Dissertation

	Studie 1[a]	Studie 2[b]	Studie 3[c]
Studienplanung	+++	+++	+++
Datenerhebung	+++	+++	+++
Datenanalyse	++	+++	+++
Verfassen des Manuskripts	++	+++	+++
Editieren des Manuskripts	++	++	++

[1]Legende: +++: > 70%
++: 50-70%
+: < 50%

[a]Evaluation of a filter system to harvest plasma for identification of failure of passive transfer in newborn calves

[b]Evaluation of 2 different treatment procedures after calving to improve harvesting of high-quantity and high-quality colostrum

[c]Evaluation of different analytical methods to assess failure of passive transfer in neonatal calves

Berlin, 28. Juli 2020 Franziska Sutter